IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Carl W. Anderson, et al.**

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For: **DNK-PK ASSAY**

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132 IN ACCORDANCE WITH *IN RE KATZ*

Sir:

I, Carl W. Anderson, declare as follows:

1. I am the inventor of the patent application identified above.

2. My current position is Chairman and Senior Geneticist in the Biology Department, at Brookhaven National Laboratory, Upton, NY 11973.

3. I am a co-author of a peer-reviewed research paper published in *Molecular and Cellular Biology* **12**(11):5041-5049 (1992), which was cited by the Examiner in the Office Action of June 19, 2003. This research paper names Susan P. Lees-Miller, Kazuyasu Sakaguchi, Stephen J. Ullrich, Ettore Appella and myself as co-authors.

4. Susan P. Lees-Miller, Ph.D. was Postdoctoral Fellow working in my laboratory from February 17, 1987 until February 1, 1990, and then an Assistant Scientist

in my research group in the Biology Department, Brookhaven National Laboratory from February 1, 1990 until August 12, 1992. Dr. Lees-Miller, who is now a Professor in the Department of Biochemistry & Molecular Biology at the University of Calgary, Canada, did not contribute to the conception of the composition for detecting DNA-PK activity in a biological sample and of a kit for detecting the presence of DNA-PK activity in a biological sample. Dr. lees-Miller contributed her expertise under my direction and supervision in enzyme purification and performing protein kinase assays.

5. Kazuyasu Sakaguchi, Ph.D., was a Staff Scientist in the Laboratory of Cell Biology at NCI, National Laboratory, and currently is Professor, Laboratory of Biochemistry, Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060_0810, Japan; he did not contribute to the conception of the composition for detecting DNA-PK activity in a biological sample and of a kit for detecting the presence of DNA-PK activity in a biological sample. Kazuyasu Sakaguchi contributed his expertise, under my direction and supervision, in synthesizing and purifying peptides.

6. Stephen J. Ullrich, Ph.D., was a Postdoctoral Fellow in the laboratory of Dr. Ettore Appella, NCI, NIH, Bethesda, MD and later became a Staff Scientist at Human Genome Sciences, Inc., and did not contribute to the conception of the composition for detecting DNA-PK activity in a biological sample and of a kit for detecting the presence of DNA-PK activity in a biological sample. Dr. Ullrich contributed his expertise under my direction and supervision in conducting experiments involving immunological assays.

7. Ettore Appella, Ph.D., was Chief Scientist, Chemistry Section Laboratory of Cell Biology at the National Cancer Institute at the National Institutes of Health, and did not contribute to the conception of the composition for detecting DNA-PK activity in a biological sample and of a kit for detecting the presence of DNA-PK activity in a biological sample. Dr. Appella supervised work, under my direction, at the National Institutes of Health related to the synthesis and purification of synthetic peptides and performing immunological analyses.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Dated: Sept 19 2003

Carl W. Anderson

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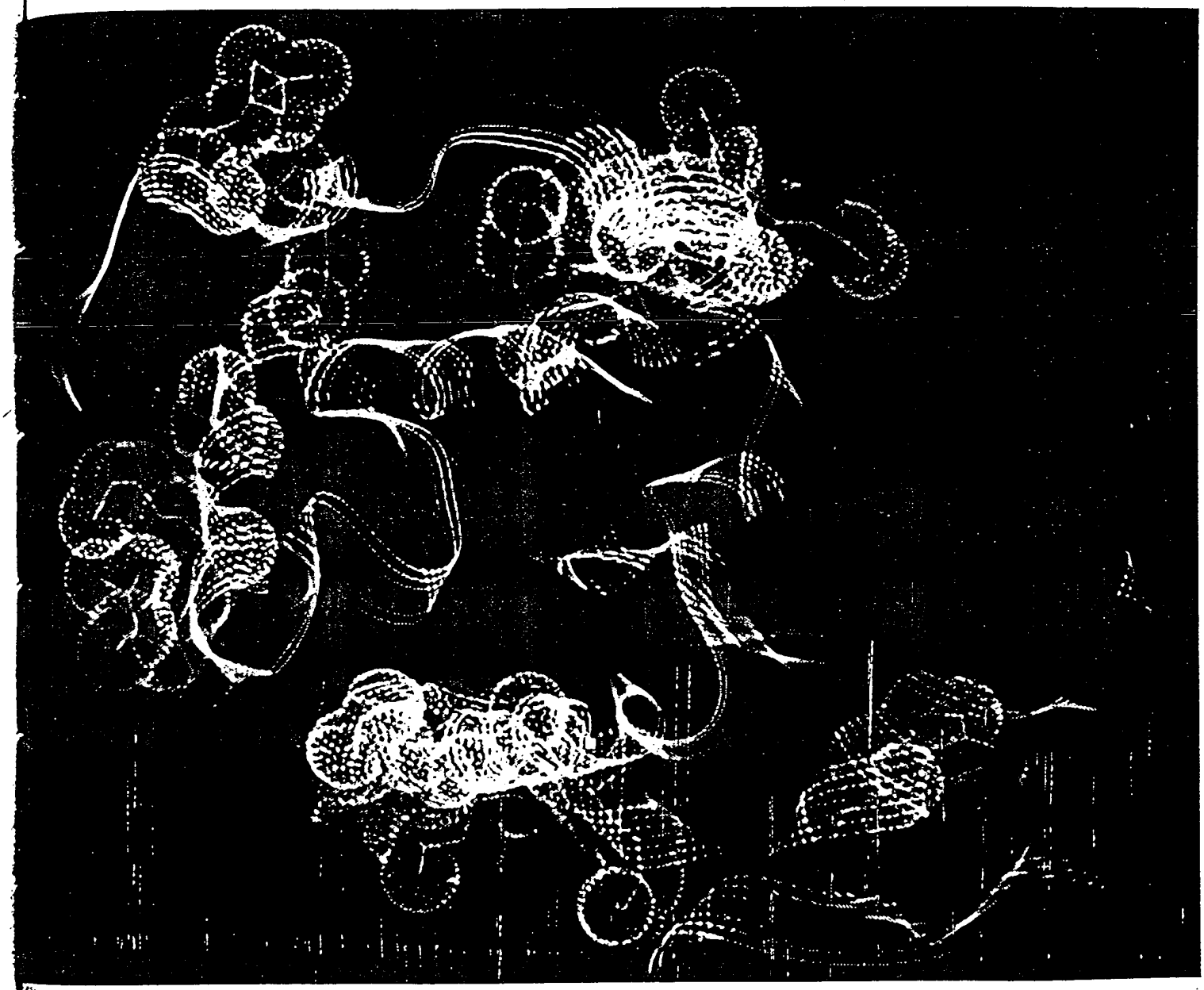

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52. Carl W. Anderson,[1] Marjorie A. Connelly,[1] H ng Zhang,[1] John D. Sipley,[1] Susan P. Lees-Miller,[1] Kazuyasu Sakaguchi,[2] Stephen J. Ullrich,[2] Stephen P. Jackson,[3] and Ettore Appella.[2] The Human DNA-Activated Protein Kinase, DNA-PK, Is Activated by DNA Breaks and Phosphorylates Nuclear DNA-Binding Protein Substrates on Serines and Threonines Following by Glutamine. ([1]Biology Department, Brookhaven National Laboratory, Upton, New York 11973; [2]Laboratory of Cell Biology, National Institutes of Health, Bethesda, Maryland 20892; [3]JCRC/Wellcome Research Institute, Cambridge CB2 1QR, England)

Eukaryotic cells respond to DNA damage by activating the expression of genes presumed to be involved in repairing damage and by interrupting cell cycle progression in the G_1 or G_2 phase of the cell cycle. The biochemical mechanisms that recognize damaged DNA and transmit signals to the cell cycle engine and transcription apparatus have not been identified. While cells may use several mechanisms to respond to DNA damage, studies in both yeast and mammalian cells suggest that one DNA damage signal is DNA strand interruption. Recently, exposure of cells to DNA-damaging agents was shown to activate several protein kinases (Anderson, 1994).

We have identified a protein kinase, DNA-PK, in extracts of human cells that may function as a DNA strand interruption detector (Lees-Miller *et al.*, 1990). Purified human DNA-PK is activated several hundredfold by double-stranded DNA fragments. Two subunits of human DNA-PK have been identified that are required for activity. One is a very large polypeptide (450 kD) that we call Prkdc. The Prkdc polypeptide is labeled by ATP analogs, suggesting that it might contain the catalytic domain (Lees-Miller *et al.*, 1990). The second required subunit is a DNA-binding protein called Ku (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993) that was originally identified as an autoantigen in patients suffering from lupus and scleroderma overlap syndrome. Ku is composed of 70- and 80-kD polypeptides that form a heterodimer (p70/p80) and appears to function as a DNA targeting/regulatory subunit that activates the catalytic subunit when Ku binds DNA structures. Ku binds well to linear duplex DNA fragments longer than about 20 bp, to nicked or gapped DNA circles, and to certain closed DNA structures that have single-to-double-strand transitions; however, Ku binds poorly to short duplex DNA fragments and to covalently closed DNA circles (Falzon *et al.*, 1993). These findings are consistent with a role for DNA-PK in detecting DNA strand interruptions that may result from DNA damage or from normal nuclear processes associated with transcription, replication, and recombination.

In vitro DNA-PK phosphorylates a variety of DNA-binding proteins including transcription factors (e.g., p53, Sp1, Fos, Jun, SRF, Myc), the carboxy-terminal repeat domain (CTD) of the large subunit of RNA polymerase II, replication protein A, and the large T-antigen of simian virus 40 (Anderson, 1994*a*). Fos, Jun, SRF, and p53 are involved in the mammalian DNA damage response (Anderson, 1994*b*). Direct peptide sequencing was used to identify DNA-PK phosphorylation sites in the heat-shock protein hsp90, in SV40 T-antigen, in the p53 tumor suppressor protein, and in the serum response factor SRF; a genetic approach was used to identify the phosphorylation site in Jun (Bannister *et al.*, 1993). Each identified site is a serine or threonine followed immediately by a glutamine. Peptides corresponding to potential -SQ- and -QS-sites in human p53 were screened for substrate activity, and three were phosphorylated well (Lees-Miller *et al.*, 1992). One, corresponding to the amino-terminal 24 residues of human p53, was analyzed in more detail, and serine 15 was identified as the residue phosphorylated by DNA-PK. Changing Thr-18 and Ser-20 in the sequence $EPPSQETFTLDWK^{24}K$ to alanine had little effect on substrate activity, but shortening the peptide to less than ten residues on the carboxy-terminal side of Ser-15 was detrimental. Changing Gln-16 to Asn, Tyr, or Glu also was detrimental, and inverting Gln-16 and Glu-17 abolished peptide substrate activity. Deleting amino acids to the amino-terminal side of Ser-15 improved substrate activity slightly; a

peptide with only two amino acids N-terminal of the serine phosphorylation site is the most active peptide substrate thus far identified. For proteins, a second specificity determinant appears to be DNA binding. *In vitro*, colocalization of substrate and kinase on DNA fragments can produce a local elevation in concentration that can be substantial.

Non-SQ/TQ-sites are phosphorylated in some substrates, but the basis for their recognition is unknown.

cDNAs for the Ku polypeptides were cloned and sequenced before Ku was known to be a DNA-PK subunit, and recently both human genes were mapped (Cai *et al.*, 1994). Protein sequence data was used to develop probes for cloning the Prkdc cDNA, and clones corresponding to more than 13 kbp have been obtained (K. Hartley *et al.*, unpublished data). Clones analyzed to date reveal an open reading frame that is substantially longer than 3000 codons. Several *PRKDC* fragments have been cloned, and the *PRKDC* gene has been mapped by *in situ* hybridization to chromosome 8. A preliminary exon/intron analysis suggests the gene could be 130 kbp in length and may contain as many as 100 exons (J. D. Sipley *et al.*, in preparation). An assay that will detect DNA-PK activation *in vivo* is being developed.

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53. Y ng-hong Xie, Manlan Yang, Jun A. Quion, Antoni M. Gotto, Jr., and Chao-yuh Yang. Quantitative Determination of Lip pr tein Particles Containing Ap lipoprotein B and E in Plasma by an Enzyme-Linked Immunos rbent Assay. (Department of Medicine, Baylor College of Medicine and Methodist Hospital, Houston, Texas 77030)

Plasma lipoproteins consist of a mixture of particles which can be differentiated by their protein composition, and contain one, two, or more apolipoproteins (apos) associated with lipid (Alaupovic *et al.*, 1972). These complex particles are generally subdivided into two major classes, particles containing apo B [including low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), and very low-density lipoproteins (VLDL)] and particles containing apo A-I [high-density lipoproteins (HDL)]. Immunological methods with two site differential immunoenzymatic assays have been developed for measuring the particles containing two or three different apolipoproteins (Kandoussi *et al.*, 1991, Sandkamp *et al.*, 1992). In this study, we report the establishment of the quantitative determination of Lp B:E and Lp E:B particles in plasma and obtain the mole ratio of apoE to apoB in the particles that contained apoE and apoB in plasma.

Samples of fasting human plasma were randomly collected from the Methodist Hospital Lipid Clinic Laboratory and divided into four groups based on the triglyceride (TG) and total cholesterol (TC) levels. Plasma were either stored at 4°C and anlyzed within days or frozen at −20°C until assayed. Purified human LDL and apoE were used as antigens to raise polyclonal antibodies. Omega Lipid Fraction Control Serum was used as a standard for apoB and apoE quantitation.

To determine apoE in apoB-containing particles (Lp B:E) in plasma, 96-well polystryene ELISA plates were coated with affinity-purified goat anti-apoB antibodies. After addition of samples and standards, goat anti-apoE conjugate was pipetted. To determine apoB in apoE-containing particles (LpE:B) in plasma, all procedures were the same as for LpB:E particles determination, except that goat anti-apoE antibodies were coated and goat anti-apoB conjugate was used.

Primary standards prepared from human fasting plasma were isolated by immunoaffinity chromatography. For Lp B:E assay, the retained fractions of an anti-LDL column were used as a primary standard, which was calibrated by Omega Lipid Fraction Control Serum (Omega Standard) to quantiate its apoE concentration. This gave us the amount of

apoE associated with apoB (Lp B:E). The apoE concentration in primary standard was used to determine the concentration of Lp B:E particles in Omega standard, which was used as secondary standard. For Lp E:B assay, the retained fraction of an anti-apoE column was used as primary standard that was calibrated by Omega Standard to determine its apoB concentration. This primary standard was used to determine the concentration of Lp E:B particles in Omega Standard, which was used as secondary standard.

According to TG and TC levels, 126 samples were divided into four groups: A, normolipidemic group (TG < 200 mg/dl, TC < 240 mg/dl); B, hypertriglyceridemic group (TG ≥ 200 mg/dl, TC < 240 mg/dl); C, hypercholesterolemic group (TG < 200 mg/dl, TC ≥ 240 mg/dl). D, combined hyperlipidemic group (TG ≥ 200 mg/dl, TC ≥ 240 mg/dl). The TG and TC means for groups A, B, C, and D were 101, 339, 119, and 311 mg/dl, and 184, 216, 266, and 300 mg/dl, respectively. As expected, the three hyperlipidemic groups showed elevated plasma concentration of apoB and Lp E:B as compared to the normolipidemic groups due to higher TC levels of hyperlipidemic groups. Groups B and D revealed a significant increase in apoE and Lp B:E as compared to groups A and C, based on the analytical results of Lp B:E and Lp E:B concentration; the mole ratio of apoE to B for lipoprotein particles containing apoE and apoB in plasma was calculated to be 1.86, 2.11, 1.70, and 1.95 for groups A, B, C, and D, respectively.

In conclusion, TG level in plasma was correlated with apoE and Lp B:E concentration in plasma. The E/B ratio for particles that contain apoE and apoB in plasma was around 2; however, higher TG levels may shift E/B to higher ratios. This method can be directly used to determine E/B ratio for particles that contain apoE and apoB in plasma without any further purification of lipoprotein particles. These data many provide important information in the study of lipid metabolism for understanding how apoE is distributed in apoB-containing particles (LDL, IDL, and VLDL) and in apo A-I-containing particles (HDL).

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54. W. F. Brandt and H. Alk. Construction of a Novel and Simple Metering Valve for Pulse- and Gas-Phase Sequencing. (Biochemistry, University of Cape Town, Cape Town, South Africa)

Automated protein sequence analysis relies on the addition of reproducibly metered quantities of reagents to the protein-containing reaction cartridge for efficient cyclic degradation. The quantitative delivery of a reagent is presently performed by either a timed addition or the use of a metering loop.

We have designed a miniaturized metering membrane pump that can deliver variable small amounts of a particular reagent reproducibly. The pump is a pneumatically activated positive displacement membrane pump working in conjunction with a pneumatically activated chemical delivery valve (Brandt *et al.*, 1984). The membrane has a single check-valve fitted in the incoming line from the reagent reservoir. The membrane is activated by vacuum for the filling stroke and pressure for the pump stroke. The check-valve allows the reagent (kept under a slight positive pressure) to enter the space under the membrane at the filling stroke. At the pump stroke the reagent is forced into the chemical delivery line via the closed chemical delivery valve. The appropriate membrane acts as a pressure relieve valve and allows the content of the membrane pump to flow into the delivery line via the chemical delivery valve. The pump ($2 \times 2 \times 2$ cm) is constructed from Kel-F, a sapphire seat, and a ruby ball. It can reproducibly deliver down to 2 μl of reagent per pump stroke. The design allows the delivery of a reagent on a metered or timed basis. The amount of reagent can be regulated by adjusting the pneumatically activated stroke of the pump ranging from 2 to 6 μl and the number of pump strokes, e.g., three pump strokes 6 μl each for a total addition of 18 μl. Both the delivery and pump valves have been redesigned to abolish gas diffusion into reagent and solvent lines. The positioning and

design of the pump maintains a zero-dead-volume system. Details of the construction and its performance will be presented.

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55. R. Bhaskaran,[1] Chin Yu,[1] and C. C. Yang.[2] S lution Structures and Functional Implications of the Toxins from Taiwan Cobra Venom, *Naja naja atra*. ([1]*Department of Chemistry and* [2]*Institute of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan*, 300)

Functional variations in the homologous toxins from Elapidae venom are well known despite their sequence homologies. Taiwan cobra venom, *Naja naja atra*, consists of toxins such as cobrotoxin (NTX) and cardiotoxins (CTXs) with 60–62 amino acids. NTX blocks neuromuscular transmission by binding with acetylcholine receptor, while the action of CTXs result in various effects, such as hemolysis, cytotoxicity, and depolarization of excitable membranes. However, activity variations are noted among CTXs (Dufton and Hider, 1988). Knowledge about the conformation of these toxins in solution might be informative in order to understand the modes of action of the toxins at the molecular level. In order to understand the varied functions of the toxins, an overall comparison was undertaken and their structural features are discussed.

Solution structures of the toxins NTX, CTX II, and CTX III from *Naja naja atra* and the crystal structures of erabutoxin b and CTX V_4^{II} from *Naja mossambica mossambica* were considered for this study (Low and Corfield, 1986; Rees *et al.*, 1990; Yu *et al.*, 1993; Bhaskaran *et al.*, 1994*a*, *b*). Essentially, in all the toxins, the disulfide linkages hold the polypeptide strands together at the top, the spatial region called the central core of the molecule; three loops emerge from this globular head and five strands form part of these loops with the formation of doubly and triply stranded β-sheets. The leftmost loop (loop 1) involves the doubly stranded sheet, the middle loop (loop 2) corresponds to strands 3 and 4, whereas the rightmost loop (loop 3) involves an exposed segment and strand 5. Despite observation of overall similarity in all the toxins with regard to the central core, major structural distinctions exist in the tip of all loops.

In NTX, loops 2 and 3 contain functional residues (D25, K27, W29, D31, R33, and K47) to bind to the receptor (Harvey, 1985). The electrostatic interactions of the functional sites with the receptor cause a strong binding. This aspect has been proved from the calculations of electrostatic potentials for the molecule. In CTX, with respect to its triply stranded sheet, the average plane corresponding to it defines concave and convex sides. The observation of hydrophobic residue stretches occurring on the surface of the molecule (flanked by basic residues) is characteristic of CTXs. The orientation of nonpolar side chains for the formation of two hydrophobic clusters is unique among CTXs. Thus the formation of hydrophobic clusters and the orientation of basic residues in the neighborhood of the clusters are expected to play major roles in the functioning of CTX. The hydrophobic stretch of loop 1 is expected to penetrate the lipid phase of the membrane and to form a hydrophobic domain inside the bilayer. During this stage, CTX has a transition from edgewise to flat orientation that may result in disorganization and hence structural perturbation of the membrane (Harvey, 1985). In a recent report on the analysis of side-chain organization of a snake CTX from *Naja nigricollis*, the existence of a possible phospholipid binding site was suggested (Gilquin *et al.*, 1993). Thereby, side chains of three conserved lysines (12, 18, 35) orient in such a way that they form a cationic site to accommodate the binding of a phosphate ion found in the crystal structure of CTX V_4^{II}. In addition, the hydrophobic cluster constitutes a possible binding site for the hydrophobic moiety of phospholipids. The above possibilities are actually observed for CTX from *Naja naja atra*, duly supporting their model.

As to the functional variation between CTX and NTX, the following are expected to be the reasons. The concavity of CTX and NTX differs. The stretch of hydrophobic residues spans the tip of loop 1 in CTXs but not in NTX. The variable sites between CTX II and CTX III are distributed at the tip of the middle loop, making CTX III more hydrophobic. In addition, the cationic site of K31 in CTX III may also be responsible for its enhanced depolarizing activity (Lauterwein and Wüthrich, 1978). As the length of β-strands is small in CTX II,

the tip of its middle loop is more disordered than that of CTX III. Thus the specific characteristics of CTX molecules, namely the stetch of hydrophobic residues at the surface of the loops surrounded by basic residues, the orientation of basic residues (Lys) on one side of the molecule for the formation of a cationic site, and the formation of two distinct sides with a specific distribution of residues, are observed to be in common in CTXs to implement the common activities. The hydrophobicity of the middle loop and the number of cationic sites decide the varied depolarizing activity in CTXs.

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56. Agnes H. Henschen. Human Fibrinogen Occurs as over 1 Million Nonidentical Molecules. (Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717-3900)

Traditionally, proteins have been regarded as well-defined, uniform molecules, where one individual molecule is virtually identical to the next. This notion has been supported by the fact that the highly efficient protein primary structure analysis by prediction from the DNA sequence will result in a well-defined, unique amino acid sequence. However, information is accumulating about protein heterogeneity and posttranslational moidfications and about the functional implications of the structural variation (Krishna and World, 1993). Human fibrinogen may serve as an extreme example of a protein existing in a multitude of structural forms, many of which have been demonstrated to differ in functional properties.

Fibrinogen is a central protein in the blood coagulation system, as it is the precursor of fibrin, which forms the blood clot. The 340-kD molecule is composed of three pairs of nonidentical peptide chains, denoted Aα, Bβ, and γ, and the chains are interconnected by 29 disulfide bridges. The most common forms of the three chains in the human protein contain 610, 461, and 411 amino acid residues, respectively (Henschen and McDonagh, 1986).

Fibrinogen occurs in so many different molecular forms because there are several sites or sections of the molecule which can exist as one of two or more structural alternative forms and these regional variants may be combined in various ways. The number of combinations is especially large, as the molecule is dimeric and each alternative regional form may occur on both sides, one side, or neither side of the molecule. Regional variants can belong to either of two categories, those which are noninherited and may be present in all individuals and those which are inherited and therefore present only in certain individuals.

Three principal types of noninherited regional variants are present in mammalian fibrinogen. They are caused by alternative splicing, by postranslational modification of specific amino acid residues, and by proteolytic degradation, respectively. The C-terminal regions of the Aα and γ chain occur in two splice-variant forms. In the Aα chain the abundant form contains 610 residues and a rare form carries an extension of 237 residues. In the γ chain the last 4 residues out of 411 in the more common form are replaced by a stretch of 20 residues in the less common form.

Two serine residues in the Aα chain are partially phosphorylated, the degree of phosphorylation depending on biosynthesis rate and molecular age. Many mammalian fibrinogens contain fully or partially sulfated tyrosines in the N-terminal region of the Bβ chain. The human protein has two fully sulfated tyrosines close to the C-terminus of the longer γ chain. A certain proline residue of the Bβ chain is partially hydroxylated. All three peptide chains seem to be subject to partial oxidation of certain methionine residues. There are two asparagine residues, one in the Bβ and one in the γ chain, which are fully glycosylated, but the

carbohdyrate side-chain structure may differ in health and disease, especially the number of terminal sialic acid residues. Amino groups modified by glucose addition are found in diabetic individuals.

Proteolytic degradation affects the Aα and γ chains in all individuals, so that the 340-kD form partially is processed to several forms of about 305 and 270 kD. In all cases, large C-terminal portions of the chains are removed by unidentified enzymes. The ratios among the various forms differ in health and disease.

Inherited variants can be either common or very uncommon in a population. The common genetic variants and the corresponding polymorphic sites give rise to sequence microheterogeneity in pooled samples. Evidence for polymorphism has been found for one site in the Aα and one in the Bβ chain. The one in the Bβ chain seems to be related to the property of fibrinogen as a risk factor in thromboembolic disease. The uncommon genetic variants have so far only been described in association with fibrinogen-related diseases.

It may be summarized that normal human fibrinogen contains at least 7 variant sites in the Aα chain, 4 in the Bβ chain, and 6 in the γ chain, i.e., a total of 17 sites. Each of the corresponding alternative forms may be symmetrically or unsymmetrically distributed in the fibrinogen molecules, but one or two of the sites may occur in genetically homozygous form. From this it can be calculated that each individual would carry over 1 million combinations of nonidentical fibrinogen molecules in the blood. However, this calculation is only based on the variants sites identified so far. Structural variants may often be detected only with difficulty, as they sometimes are present only in a minor part of the molecules, they sometimes are structurally labile, and specific methods for their detection are lacking. Thus, tryosine sulfate may escape attention, as it is converted to unmodified tyrosine during sequencing, and hydroxy-proline may remain unidentified, as the modification is partial. In addition, no quantitative identification methods have been developed for these modified amino acids.

The various molecular forms are likely to differ considerably in their functional properties (Henschen, 1993). Obviously, it is virtually impossible to separate all these forms, but specific procedures can be used to fractionate the molecular population according to certain structural features so that the functional relevance may be tested.

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57. Keith Ashman. Preelectrophoretic Labeling of Proteins with a Colored Water-Soluble Edman Reagent. (Centre for Animal Biotechnology, School of Vertinary Science, University of Melbourne, Parkville 3052, Melbourne, Victoria, Australia)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is still the most powerful method of resolving a complex mixture of proteins. However, its major use is still as an analytical rather than a preparative tool. With the advent of PVDF membranes which are stable under the conditions employed in the Edman degradation, it has become common to try and obtain N-terminal sequence data from proteins separated by SDS–PAGE followed by electrophoretic transfer to a PVDF membrane. It is also possible to electroelute proteins out of gel slices for sequencing or enzymic digestion. Alternatively the proteins can be enzymically digested within the gel matrix and the peptides eluted for subsequent HPLC purification. Both these procedures require the proteins to be stained after electrophoresis in order to locate their position in the gel. The staining process generally fixes the proteins in the gel and leads to signficant loss of material.

Some of these problems may be overcome by preelectrophoretic labeling (Kraft *et al.*, 1988). A simple method of prelabelling proteins with a water-soluble Edman reagent S-DABITC (Chang, 1989) which couples to the N-terminal amino acid and the epsilon amino group of lysine has been developed. The reaction takes place under very mild conditions and the reagent has been described for its use in the identification of reactive lysines on the surface protein molecules (Chang *et al.*, 1992). By denaturing proteins in the presence of SDS it is possible to label all the available sites on a molecule. This provides a simple method of generating colored marker proteins for electrophoresis which can be used in preparative electrophoresis apparatus or on SDS–PAGE gels. More importantly, the labeled proteins can still be sequenced after the labeling

procedure and electrophoretic separation. The N-terminal label is removed during the first cycle of Edman degradation. The labeled molecules can either be transferred to a suitable membrane for direct sequencing or passively eluted from the gel, since no fixing or further staining is required, and collected on a Prospin cartridge or similar device. Passive elution is especially useful for high-molecular-weight proteins, where it is often necessary to collect material from several gels to obtain enough for sequencing. The fact that the proteins carry a colored label makes it easier to keep track of them. Further, the label does not interfere with enzymic or chemical digestion and lysine-containing peptides are readily identified during HPLC separation because they have a characteristic absorption at 450 nm. The procedure has been tested on several proteins and found it to be a practical method of labeling and recovering proteins and peptides for sequencing.

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58. Matthias Mann. Role of Mass Accuracy in the Identification of Proteins by Their Mass Spectrometric Peptide Maps. (European Molecular Biology Laboratory, Heidelberg, Germany)

Characterization of small amounts of proteins remains a bottleneck in molecular biology. Traditionally, proteins are purified and either N-terminally sequenced or, more usually, digested, and peptides are sequenced by Edman degradation. The resulting sequences can be used to identify the protein in a database or to construct oligonucleotide probes to sequence the corresponding DNA. Disadvantages of this approach are its limited sensitivity of 10–100 pmol, its limited throughput, which precludes the large-scale analysis of proteins, and the need to sequence a substantial part of a protein to be sure that not just its family has been identified.

We have recently investigated an alternative approach using mass spectrometry and sequence databases (Mann *et al.*, 1993). The basic idea is to correlate mass spectrometric data—which today is orders of magnitude easier to obtain compared to even 5 years ago—with the sequences in the databases—which are likewise increasing by orders of magnitude, in this case because of the human and associated genome projects.

We have investigated three kinds of mass spectrometric information in this context: (i) the complete molecular weight of the protein, (ii) the masses of peptides obtained by a sequence-specific protease or chemical reagent, and (iii) the mass of a single peptide combined with partial sequence information provided by MS/MS data or by Edman degradation. The three methods are of differing generality, with (i) being applicable only in a few specialized cases and (iii) being the most powerful and general.

In all these approaches the crucial parameter is the mass accuracy, because the mass is the parameter by which one selects. However, the role of the mass accuracy has not yet been investigated systematically. In fact, some programs written to search databases do not allow a very high mass accuracy to be enetered. In this contribution preliminary data on the effect of mass accuracy on the selectivity of database searches is investigated.

The program used in this investigation (PeptideSearch) was written for the Macintosh computer and features very flexible searches (limit digestion or consideration of one or two missed cleavages), many specific proteases, and the ability to define rules for digestion. This flexibility is provided by a very fast "on the fly" digestion which also allows search by partial sequence information on the same file. As an example, search times on SWISSPROT (ca, 30,000 proteins) are less than 20 sec on a Quadra type Macintosh computer.

Experimental results used to illustrate the search specificities were taken with a Bruker REFLEX mass spectrometer using matrix assisted laser desorption/ionization (MALDI).

When searching by the molecular weight of the intact protein, improved mass accuracy has the expected result, i.e., a proportional increase in search specificity. In contrast, when searching by the molecular masses of several peptides the effect of the mass accuracy seems to be multiplicative with the number of peptides used in the search. In this connection, recent progress in mass accuracy with MALDI time-of-flight mass spectrometry is particularly interesting (Vorm *et al.*, 1994; Vorm and Mann, 1994). Mass accuracy in complex peptide mixtures

Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein

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The p53 tumor suppressor gene product is a transcriptional activator that may be associated with its ability to suppress tumor cell growth. The acidic amino terminus of the p53 protein has been shown to contain this *trans*-activation activity as well as the domains for mdm-2 and adenovirus 5 E1B 55-kD protein binding. An extensive genetic analysis of this amino-terminal p53 domain has been undertaken using site-specific mutagenesis. The results demonstrate that the acidic residues in the amino terminus of p53 may contribute to, but are not critical for, this *trans*-activation activity. Rather, the hydrophobic amino acid residues Leu-22 and Trp-23 of human p53 are both required for *trans*-activation activity, binding to the adenovirus E1B 55-kD protein and the human mdm-2–p53 protein in vitro. In addition, hydrophobic residues Leu-14 and Phe-19 are crucial for the interactions between p53 and human mdm-2 (hdm-2). Hydrophobic residues Trp-23 and Pro-27 are also important for binding to the adenovirus 5 (Ad5) E1B 55-kD protein in vitro. These mutations have no impact on the ability of the p53 protein to bind to a p53-specific DNA element. These results suggest that 2–4 critical hydrophobic residues in the amino-terminal domain of the p53 protein interact with the transcriptional machinery of the cell resulting in transcriptional activation. These very same hydrophobic residues contact the hdm-2 and Ad5 E1B 55-kD oncogene products.

[*Key Words:* p53 protein; *trans*-activation; mdm-2 binding; E1B 55-kD binding]

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Wild-type p53 protein is the product of a tumor suppressor gene that has the ability to suppress oncogenic transformation (Eliyahu et al. 1989; Finlay et al. 1989), negatively regulate cell cycle progression (Baker et al. 1990; Diller et al. 1990; Martinez et al. 1991), and induce apoptosis in certain cell types (Yonish-Rouach et al. 1991; Shaw et al. 1992). Wild-type p53 protein has been shown to possess a transcriptional activation function (Fields and Jang 1990; O'Rourke et al. 1990; Raycroft et al. 1990) that may be linked to its tumor suppression function because many mutant p53 proteins found in human cancers have lost both *trans*-activation activity (Raycroft et al. 1991; Unger et al. 1992) and tumor suppressor activity (Hinds et al. 1990; Chu 1991). The transcriptional activation domain of p53 has been mapped to the amino-terminal residues 1–42 (Unger et al. 1992), whereas the DNA-binding domain of p53 resides in residues 120–290 (of 393 amino acids). The amino-terminal domain is rich in acidic amino acids, and it has been suggested that these negatively charged residues are required for the *trans*-activation function by p53. Inhibition of the *trans*-activation activity of wild-type p53 by several viral and cellular oncoproteins appears to correlate with their transforming ability. Two such oncogene products, mdm-2 (Chen et al. 1993; Oliner et al. 1993) and adenovirus 2 early 1B (Ad2 E1B) 55-kD protein (Kao et al. 1990), have been shown to bind to the amino terminus of p53. The *mdm-2* gene was originally cloned as a cellular oncogene amplified on a mouse double-minute chromosome (Cahilly-Snyder et al. 1987). Overexpression of the *mdm-2* gene in BALB/c-3T3 cells was shown to increase their tumorigenic potential (Fakharzadeh et al. 1991). The *mdm-2* gene product has also been shown to complex with p53 and inhibit p53-mediated *trans*-activation (Momand et al. 1992; Oliner et al. 1993). mdm-2 binds to the first 52 residues of p53 (Chen et al. 1993; Oliner et al. 1993).

The Ad2 and Ad5 E1B 55-kD proteins bind to p53 in transformed cells (Sarnow et al. 1982) and in vitro (Kao et

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Table 1. *Relative levels of CAT activity, human mdm2 and Ad5 E1B 55-kD protein binding affinity of the amino terminus human p53 mutants*

Position	Change from	Change to	CAT activity relative to wild-type human p53 (%)[a] p50–2CAT	CAT activity relative to wild-type human p53 (%)[a] CoSX1CAT	hdm-2-binding affinity relative to wild-type human p53 (%)[b]	E1B 55-kD binding affinity relative to wild-type human p53(%)[c]
Vector			5	4		
Wild-type p53			100	100	100	100
2,	Glu	Lys	79	72	66	84
3	Glu	Lys				
7,	Asp	His	58	64	81	
11	Glu	Lys				
13	Pro	Thr	56	63	85	141
15	Ser	Gly	300	117	66	
14,	Leu	Gln	56	41	1	61
19	Phe	Ser				
16,	Gln	Leu	219	210	124	41
18	Thr	Ile				
17,	Glu	Lys	40	41	80	228
21	Asp	His				
22	Leu	Gln	17	59	56	49
23	Trp	Ser	22	74	22	3
22,	Leu	Gln	5	8	2	6
23	Trp	Ser				
24	Lys	Thr	127	94	181	9
25,	Leu	Gln	143	34	39	18
26	Leu	His				
27	Pro	Tyr	126	73	779	4
28	Glu	Lys	56	36	162	47
31,	Val	Ser	96	95	101	35
32	Leu	Arg				
48,	Asp	His	215	85		
49	Asp	His				
61,	Asp	His	40	36	150	154
62	Glu	Lys				
17,	Glu	Lys	54	79	22	217
21,	Asp	His				
28	Glu	Lys				
7,	Asp	His	35	18	12	257
11,	Glu	Lys				
17,	Glu	Lys				
21,	Asp	His				
41,	Asp	His				
42	Asp	His				
273	Arg	His	8	6		

[a]Saos-2 cells were cotransfected with reporter and mutant or wild-type human p53 plasmids as described in Materials and methods. CAT activity was determined relative to wild-type p53. Each entry represents the average from three independent experiments. Results of mutant 22–23 are given as average from five independent experiments.

[b]The conditions used to analyze the human p53 and mdm-2 interaction were as described in Materials and methods. Results are given as average from two independent experiments.

[c]The methods used to analyze the interaction between E1B 55K and human p53 are described in Materials and methods. Results are given as average from two independent experiments.

failure to interact with the transcriptional machinery. It remains possible that codon 22–23 mutants failed to bind to DNA in the p53-responsive element. To test this, a Gal4 fusion protein was constructed with th Gal4 DNA-binding domain and the p53 amin terminal 53 residues containing the codon 22–23 mutants. When this was transfected into Saos-2 cells with a Gal4-responsive element regulating a CAT expression vector, the mutant p53 protein at codons 22–23 failed to *trans*-activate the test gene, whereas the wild-type p53 amino terminus does enhance the activity of the test gene (data not shown). These data indicate that the codon 22–23 defect in activating transcription occurs even when the mutant protein is bound to a DNA element.

Next, the p53 wild-type protein, the 14–19 mutant protein, 22–23 mutant protein, and the codon 175RH

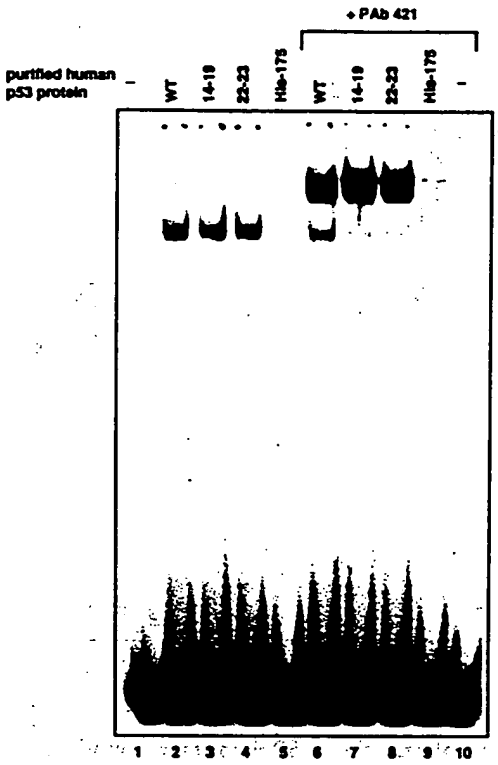

Figure 2. DNA gel shift by p53 wild-type and mutant proteins. The p53 wild-type (WT) or mutant proteins (14–19, 22–23, or His-175) were synthesized by baculovirus vectors and purified. They were incubated with a labeled DNA oligonucleotide (a p53-specific consensus sequence) and run out on the gel to look for protein binding. The oligonucleotide was incubated with no protein added (lane *1*), the p53 WT (lane 2), 14–19 mutant (lane 3), 22–23 mutant (lane *4*), and His-175 (lane *5*). p53 WT plus pAb421 antibody gives a supershift (lane *6*), as does the 14–19 mutant plus pAb421 (lane *7*) and 22–23 mutant plus pAb421 (lane *8*). The His-175 mutant fails to bind to DNA (lanes *5,9*), as well as pAb421 antibody alone (lane *10*).

structed and tested in Table 1 were surveyed for binding to the hdm-2 protein. Radiolabeled hdm-2 and mutant p53 proteins were generated by translation of these proteins in vitro. Each mutant p53 was mixed with hdm-2 protein and incubated for 30 min at 30°C. The mixture was then immunoprecipitated with a mdm-2-specific monoclonal antibody 4B2 (Chen et al. 1993; Olson et al. 1993). This antibody recognizes both human and murine mdm-2 proteins. The efficiency of forming an in vitro complex between p53 mutants and mdm-2 proteins was determined by quantitating the amount of mutant and wild-type p53 protein that coimmunoprecipitated with anti-mdm-2 antibody. An example of such an experiment is shown in Figure 5, and the results using the entire panel of mutants are summarized in Table 1. The majority of the mutants retained near wild-type levels of mdm-2-binding efficiency. A few mutants showed moderately reduced binding efficiencies (between 20 and 40% of wild type). However, double mutants Leu-14–Phe-19 and Leu-22–Trp-23 essentially failed to bind mdm-2 in vitro; the binding efficiencies range from undetectable to <5% of wild-type in individual experiments.

Identification of p53 residues required for binding to Ad5 E1B 55-kD protein

Previous experiments have demonstrated that the p53 domain that binds to the Ad2 or Ad5 E1B 55-kD protein resides in the amino-terminal 123 amino acids of p53 (Kao et al. 1990). Furthermore, the transforming ability of this oncogene product correlates with its ability to block transcriptional *trans*-activation by the p53 protein (Yew and Berk 1992). For this reason, the panel of mutants listed in Table 1 were tested for their ability to bind to the Ad5 E1B 55-kD protein. To analyze the p53/55-kD complex, the experimental conditions described by Kao et al. (1990) were used, except that the Saos-2 cells were employed instead of HeLa cells. Wild-type or mutant human p53 cells synthesized in vitro were labeled with [^{35}S]methionine and mixed with the Saos-2 cell extracts infected with wild-type Ad5. The mixture was incubated for 30 min at 30°C, and the labeled p53 proteins were coprecipitated with unlabeled E1B 55-kD protein using an anti-E1B 55-kD protein monoclonal antibody, 2A6 (Sarnow et al. 1982). The quantitative results of E1B 55-kD binding of each p53 mutant are summarized in Table 1, with these data shown in Figure 6. Most of the mutants tested showed levels of E1B 55-kD protein binding close to that of wild-type p53. A few mutants showed a two- to threefold reduction in binding efficiency. However, mutations in a small region from residues 23–27 had the poorest binding affinity to E1B 55-kD protein (Table 1; Fig. 6). In this region, mutants 23 and 27 almost failed to bind to the 55-kD protein in vitro, with binding efficiencies ~4% of the wild-type level. A tyrosine substitution at the residue Pro-27 in the wild-type protein is of some interest because it produced a p53 protein that bound very poorly to E1B 55 kD, had enhanced binding to the hdm-2 protein, and retained near wild-type levels of *trans*-activation activity (Table 1; Figs. 5 and 6). These results indicate that these mutations do not result in dramatic changes in protein conformations, but, rather, the residues involved are critical for protein–protein contacts. It appears likely that residues 23, 24, and 27 play an important role in p53–E1B 55-kD interactions.

The interactions of p53 mutants with TBP

Previous experiments have shown that wild-type p53 protein binds to TBP (Seto et al. 1992) and that the amino terminus of p53 appears to mediate this interaction (Liu et al. 1993; Truant et al. 1993). It was of some interest, therefore, to determine whether p53 mutants at 22–23 or 14–19 failed to bind to TBP. Several assays have been employed to demonstrate p53–TBP interactions, including coimmunoprecipitation of a mixture of these two proteins, a far Western blot procedure with labeled TBP binding to p53 transferred from denaturing gels to filter paper, or the cosedimentation or cochromatography of

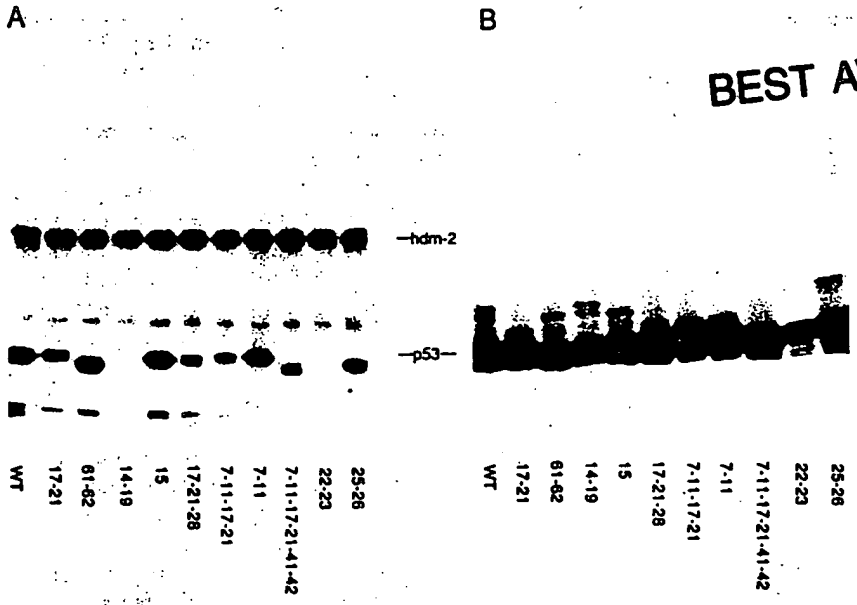

Figure 5. Ability of p53 mutants to complex with hdm-2. Hdm-2 and mutant p53 were generated by in vitro translation in rabbit reticulocyte lysates labeled with [^{35}S]methionine. Each mutant p53 protein was mixed with hdm-2 and incubated for 30 min at 30°C. The mixture was then immunoprecipitated with a mdm-2-specific monoclonal antibody, 4B2, as described in Materials and methods. (*A*) Coprecipitation of p53 mutants with hdm-2 and (*B*) in vitro translation products of p53 mutants. Two microliters of the ^{35}S-labeled in vitro translation products was separated on the SDS–polyacrylamide gel to verify the mutant protein synthesis.

amino-terminal domain is thought to contact the transcriptional machinery of the cell. The p53 protein does interact with TBP using sequences at the amino-terminal domain of p53 (Seto et al. 1992; Liu et al. 1993; Truant et al. 1993). In particular, it had been suggested that the highly acidic amino acid nature of the amino-terminal domain—there are 9 acidic residues among the first 42 residues of human p53—might mediate the interactions between p53 and some basal transcription factors (Mitchell and Tjian 1989; Stringer et al. 1990). To test these ideas, an extensive analysis, using site specific mutagenesis of the first 42 amino acid residues of the human p53 protein, was carried out.

Placing nonconservative genetic alterations in the amino-terminal domain of p53 has resulted in clear loss of function of this protein for its transcription factor phenotype, binding to the hdm-2 protein, and binding to the Ad5 E1B 55-kD protein (see Table 1). There are two ways in which such mutations could result in these loss of functions: (1) By altering the amino acid contacts between two proteins, the function is lost and the mutation identifies such contact points in a protein; and (2) by altering the conformation or stability of the entire p53 protein, a function may be lost, but the real protein contact points are not elucidated. Several lines of evidence favor the former interpretation of the results presented here. (1) Several conformational-sensitive monoclonal antibodies recognize these amino-terminal domain mutant proteins in a "wild-type" or native conformation (pAb1620) and not in a "mutant" or denatured conformation (pAb240). The mutant conformation of p53 produces a protein that fails to act as a transcription factor (Fig. 3). The mutations in the amino-terminal domain do not alter the DNA-binding domain structure as observed

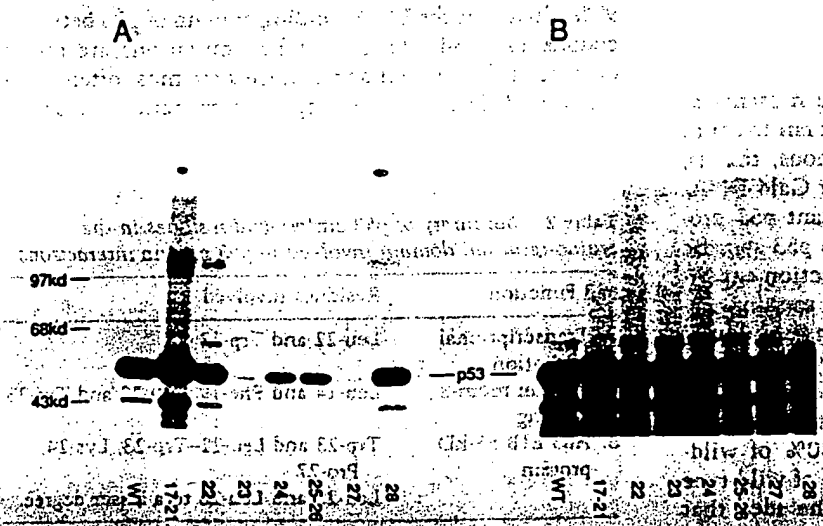

Figure 6. Ability of p53 mutants to bind Ad5 E1B 55-kD protein. Mutant or wild-type p53 synthesized in vitro was labeled with [^{35}S]methionine and mixed with whole Saos-2 cell extracts infected with wild-type Ad5. The mixture was incubated for 30 min at 30°C and precleared with protein A–Sepharose for 15 min at 4°C. The labeled mutant p53 proteins were coprecipitated with unlabeled E1B 55-kD protein by anti-E1B 55-kD protein monoclonal antibody 2A6 (Sarnow et al. 1982). The precipitated proteins were detected by 12.5% SDS–polyacrylamide gel electrophoresis and autoradiography. (*A*) Coprecipitation of p53 mutants with E1B 55-kD protein and (*B*) in vitro translation products of p53 mutants. Two microliters of the ^{35}S-labeled in vitro translation products were run on the SDS–polyacrylamide gel to verify the synthesis of mutant proteins.

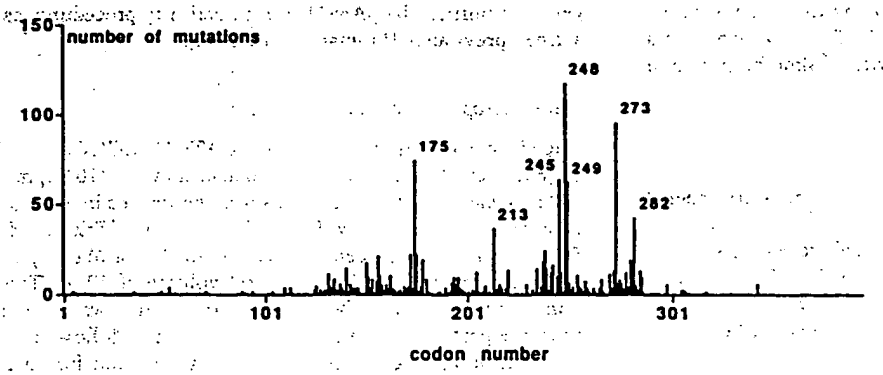

Figure 8. The codon position in the p53 gene of 1447 different mutations that have occurred in 51 different types of human cancers. The graph shows the frequency of different missense mutations as a function of the codon in the p53 gene derived from various types of cancers in humans. The results are from the Princeton University data base of such mutations (Levine et al. 1994).

298 and six at codon 342 are all (12) chain-termination mutations. Codon 53 contains four independent mutations, and three are chain-termination mutants. It is thought that these mutants result in selection against the transcriptional activity of the p53 protein (Farmer et al. 1992; Kern et al. 1992; Zambetti et al. 1992). Why, then, are all of the missense mutations clustered in the DNA-binding domain and few, if any, in the amino-terminal *trans*-activation domain? The answer (Table 1) appears to be that two independent mutations at codons Leu-22 and Trp-23 are required to have the same phenotype as one mutation in the DNA-binding domain (273RH mutant). This distribution of mutations in Figure 8 is consistent with the results presented here and reinforces the evidence that the p53 "loss-of-function" mutation is to select for the loss of a transcription factor. The fact that two oncogenes, one human (*mdm*-2) and one viral (Ad5 E1B 55-kD), target the very same amino acids used in protein contacts for transcriptional activation is also most consistent with the critical role of transcription in the function of the p53 protein as a tumor suppressor gene.

Soussi et al. (1990) have pointed out that the p53 proteins whose cDNAs have been sequenced from rainbow trout to human contain five highly conserved regions of amino acid sequences. Four of them are in the DNA-binding domain (residues 120–143, 172–182, 238–259, 271–290) and one is in the amino-terminal domain between residues 13 and 19. Residues Leu-22 and Trp-23, however, are also identical in all of the p53 proteins in this comparison (Fig. 9) and, so, the conserved region I should probably be extended from amino acid 13 to 23 to more closely reflect the functional significance of this conservation.

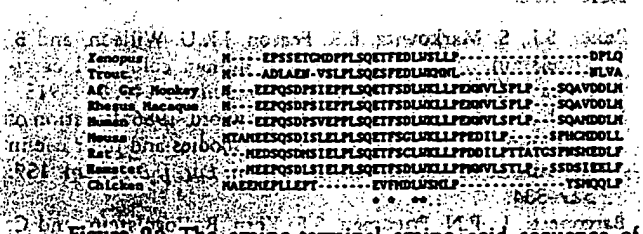

Figure 9. The amino-terminal amino acid sequence of p53 from several species. The one-letter amino acid code is used to compare the sequences of p53 proteins from diverse species. Gaps have been employed to maximize the similarities in these sequences. The last residue number is given at *right* for each sequence. Asterisks indicate identity in all sequences. Dots indicate similarities in all sequences.

Materials and methods

Plasmids and site-specific mutagenesis

pBKS–p53 consists of a Bluescript KS(−) vector (Stratagene) and the entire wild-type human p53 cDNA ligated at their *Eco*RI sites. pRC/CMVp53 consists of a pRC/CMV vector (Invitrogen) and the entire wild-type human p53 cDNA ligated at their *Hin*dIII and *Xba*I sites. Mutations were generated in pBKS–p53 or pRC/CMVp53 by annealing oligonucleotides with one or multiple mismatches to uridine-containing single-stranded DNA template as described previously by Kunkel (1985). The oligonucleotide was extended with T4 DNA polymerase, and the resulting double-stranded DNA was sealed with T4 DNA ligase. The DNA was used to transform *Escherichia coli* JM109 (Invitrogen). Single-stranded or double-stranded DNA was prepared and sequenced by the dideoxy procedure (Sanger et al. 1977) using appropriate primers. p53 mutants that generated in pBKS–p53 were cleaved with *Hin*dIII and *Xba*I recloned in these sites in the expression vector PRC/CMV.

For CAT assays, three reporters were used, p50–2CAT, CosX1CAT, and pG$_5$E1BCAT. p50–2CAT consists of two copies of p53-responsive element from the murine muscle creatine phosphokinase promoter (Zambetti et al. 1992). CosX1CAT contains a p53-responsive element from murine *mdm*-2 gene (Wu et al. 1993). pG$_5$E1BCAT contains four Gal4 DNA-binding sites, the E1B TATA sequence, and the CAT gene (kindly provided by Dr. T. Shenk, Princeton University).

Cell culture and DNA transfection

The human osteogenic sarcoma (Saos-2) cells that do not express endogenous p53 (Masuda et al. 1987) were used for CAT assays and transient expression of p53 protein. Saos-2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal bovine serum (FBS) in a 5% CO_2, 37°C incubator. For CAT assays, the cells grown on a 10-cm^2 tissue-culture dish were cotransfected with 2.5 μg of p53 plasmid DNA and 2.5 μg of reporters plus 15 μg of salmon sperm DNA by calcium phosphate protocol (Graham and van der Eb 1973). Forty-eight hours after transfection, CAT activity was assayed as described previously (Zambetti et al. 1992) and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics). For transient expression of p53 protein, Saos-2 cells grown on a 15-cm^2 tissue-culture dish were transfected with 20 μg of p53

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Immunochemical analysis of the interaction of p53 with MDM2; – fine mapping of the MDM2 binding site on p53 using synthetic peptides

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The function of p53 is modulated by binding to a number of cellular and viral proteins, such as MDM2 and SV40 large T antigen. An initial immunochemical characterization of the p53-MDM2 complex in a rat fibroblast cell line (Clone 6) suggested that the anti-p53 monoclonal antibody Bp53-19 failed to immunoprecipitate the complex, and only recognized a fraction of the available p53 protein. Following the recent identification f the Bp53-19 epitope at the N-terminal end of p53, in the vicinity of where MDM2 protein was known to bind, we investigated the possibility that Bp53-19 might identify a region of p53 that interacts with MDM2 protein. MDM2 was found to bind with great specificity to short synthetic peptides derived from the N-terminus f p53. Several p53 synthetic peptides libraries, and an alanine substitution series at the optimal binding site, were used to establish the MDM2 binding site, in fine detail, to the sequence TFSGLW (aa 18–23) in mouse and TFSDLW in man (aa 18–23). The key residues required for MDM2 binding are almost identical to those required for the monoclonal antibody Bp53-19 to bind and this region of p53 is recognised by many other anti-p53 antibodies.

Introduction

Inactivation of the p53 tumour suppressor is a frequent event in human neoplasia. The inactivation can occur by mutation of the p53 gene or through binding to viral or cellular oncogene proteins, such as the large T antigen and MDM2. While the mechanism through which wild-type p53 suppresses tumour cell growth is as yet poorly defined it is clear that one key feature of the growth suppression is the property of p53 to act as a transcription factor (Farmer *et al.*, 1992; Funk *et al.*, 1992; Kern *et al.*, 1992). Currently, considerable effort is being made to identify growth control genes that are regulated by p53 binding to sequence elements near or within these genes. A number of such genes have been identified. In cases such as the muscle creatine kinase gene (Weintraub *et al.*, 1991; Zambetti *et al.*, 1992) and a GLN retroviral element (Zauberman *et al.*, 1993) the role these genes might play in the suppression of growth control is unclear. Yet there are other examples, namely *mdm2* (Barak *et al.*, 1993; Wu *et al.*, 1993), GADD 45 (Kastan *et al.*, 1992) and WAF1 or CIP1 (El-Deiry *et al.*, 1993; Harper *et al.*, 1993), where their involvement in the regulation of cell growth is better understood.

Mdm2, a known oncogene, was originally found on *m*ouse *d*ouble *m*inute chromosomes (Cahilly-Snyder *et al.*, 1987). Its protein product was subsequently found to form a complex with p53, which was first observed in a rat fibroblast cell line (Clone 6) previously transfected with a temperature sensitive mouse p53 gene (Michalovitz *et al.*, 1990). The rat cell line grew well at 37°C but exhibited a G1 arrest when shifted down to 32°C, which was entirely consistent with an observed temperature dependent switch in p53 conformation and activity. However, the p53-MDM2 complex was only observed in abundance at 32°C, at which temperature p53 was predominantly in a functional or 'wild-type' form (Bank & Oren, 1992; Momand *et al.*, 1992). By shifting the rat cell line down to 32°C and blocking *de novo* protein synthesis it was shown that only 'wild-type' p53 induced expression of the *mdm2* gene, thereby accounting for the differential abundance of the complex in terms of p53 transcriptional activity (Barak *et al.*, 1993). The explanation was further developed by the identification of a DNA binding site for wild-type p53 within the first intron of the *mdm2* gene (Wu *et al.*, 1993). Reporter constructs employing this p53 DNA binding site revealed that they were inactivated when wild-type p53 was co-expressed with *mdm2*. This inhibition of the transcriptional activity of p53 may be caused by MDM2 blocking the activation domain of p53 and/or the DNA binding site. Consequently, it was proposed that *mdm2* expression is autoregulated, via the inhibitory effect of MDM2 protein on the transcriptional activity of wild-type p53. This p53-*mdm2* autoregulatory feedback loop provided a novel insight as to how cell growth might be regulated by p53. Since up to a third of human sarcomas are considered to overcome p53-regulated growth control by amplification of the *mdm2* gene (Oliner *et al.*, 1992), the interaction between p53 and MDM2 represents a key potential therapeutic target. We therefore sought to immunochemically characterize the p53-MDM2 complex, and also determine in fine detail the MDM2 binding site on p53.

Results

The first indication of an interaction between MDM2 protein and p53 protein emerged from work on a rat cell line, Clone 6, which expressed a temperature sensitive mutant form of mouse p53 (Michalovitz *et al.*, 1990; Barak & Oren, 1992; Momand *et al.*, 1992). MDM2 was readily observed to form a complex at 32°C with p53 but was just detectable when cells were

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grown at 37°C. Re-examining this observation with a panel of p53 monoclonal antibodies we were surprised to find that one of the antibodies, Bp53-19, failed to immunoprecipitate p53 from Clone 6 cells grown at 32°C for 24 h, but efficiently precipitated p53 from cells grown continuously at 37°C (Figure 1a and b), whereas PAb421 precipitated p53 at both temperatures (Figure 1a and b). We were therefore curious as to whether Bp53-19 would co-immunoprecipitate MDM2 with p53. From the immunoprecipitation western data in Figure 1a and b it is clear that Bp53-19 does not co-immunoprecipitate MDM2 from cell extracts grown at 32 or 37°C. Other p53 antibodies such as PAb421 do however co-immunoprecipitate MDM2 with p53 at 32°C but not at 37°C. Conversely, antibodies against MDM2 such as 4B2 (Figure 1a and b) 2A10 (data not shown) and SMP14 (data not shown) co-immunoprecipitate p53 at 32°C but not at 37°C. The two bands immunoprecipitated by 4B2 (also observed with immunoprecipitations by 2A10 and SMP14, data not shown) at just below 80 kDa are truncated forms of rat MDM2, as the full length form migrates on an SDS–PAGE gel with an apparent relative molecular mass of 95 kDa. The same two bands immunoprecipitated by 4B2 were also detected by another anti-MDM2 monoclonal antibody, 2A10, again only in extracts prepared from cells grown at 32°C (data not shown). MDM2 expression is complex since there are multiple forms of MDM2, with relatively short half-lives of approximately 20 to 30 min (Barak *et al.*, 1993; Olson *et al.*, 1993). The immunoprecipitation observations are consistent with two-site immunoassays of the levels of MDM2, p53 and MDM2-p53 complex at 32°C and 37°C (Figure 2a and b). A striking feature apparent from the data in Figure 2a is that the levels of p53 and p53-MDM2 complex are very similar, suggesting that most, but not all, p53 is in complex with MDM2 at 32°C. The inability of Bp53-19 to detect a p53-MDM2 complex at 32°C is again notable since other combinations of antibodies are able to do so.

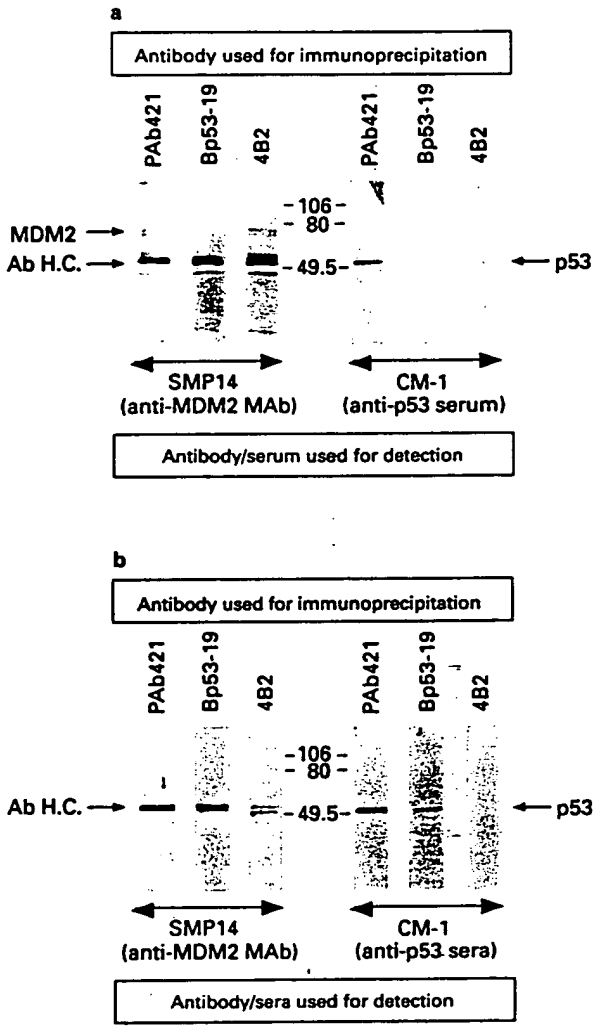

Figure 1 Western blots of immunoprecipitates of MDM2, p53 and the MDM2-p53 complex from Clone 6 cells grown at 32°C for 24 h (a) or continuously at 37°C (b). The immunoprecipitates were obtained using 1 μg of the stated purified antibody. MDM2 was detected using SMP14 antibody supernatant and a rabbit anti-mouse horseradish peroxidase conjugate, and p53 was detected using a 1 in 200 dilution of polyclonal serum CM-1 and a swine anti-rabbit horse radish peroxidase conjugate. An irrelevant antibody, PAb419, did not immunoprecipitate either MDM2 or p53 from cell extracts prepared at either 32°C or 37°C (data not shown). The molecular weights of the markers are given in kDa. The positions of MDM2 and p53 on the western blots are indicated by arrows. The abbreviation Ab H.C. indicates the antibody heavy chain of the monoclonal antibody used in the immunoprecipitation step and is detected by the rabbit anti-mouse horse radish peroxidase conjugate rather than the monoclonal antibody SMP14.

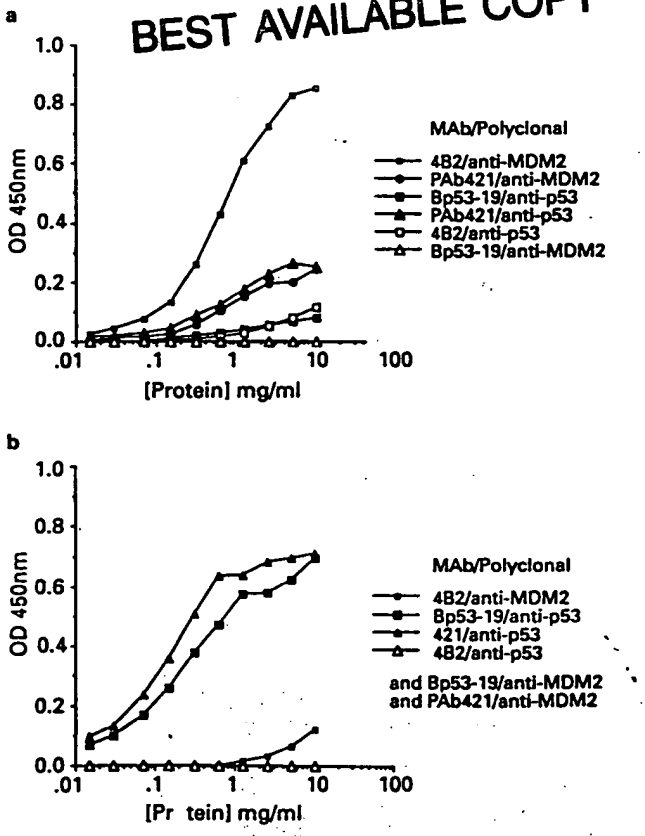

Figure 2 Two-site immunoassays to determine the levels of MDM2, p53 and MDM2-p53 complex in Clone 6 cells grown at 32°C for 24 h (a) or continuously at 37°C (b). In a, the coating antibodies were one of the following purified antibodies as shown in the figure: 4B2, PAb421 and Bp53-19, probed with rabbit anti-p53 serum CM1 or rabbit anti-MDM2 serum, and then detected using a swine anti-rabbit horse radish peroxidase conjugate and TMB as substrate. At 37°C the MDM2-p53 complex was undetectable by any combination of antibodies.

From comparison of the two-site immunoassays at 32°C and 37°C it is clear why MDM2 is not immunoprecipitated at 37°C, as the level of MDM2 protein is very much lower and is only just detectable. No MDM2-p53 complex could be detected by the two-site immunoassay of cell extracts prepared at 37°C, (see Figure 2b) where the data for the 4B2 (as the capture antibody) and CM1 (as the detecting antibody) combination of antibodies is shown (similarly antibodies PAb421 or Bp53-19 and rabbit anti-MDM2 polyclonal did not detect the complex). The diminished level of MDM2 at 37°C, less than 10% of that at 32°C, is in contrast to the situation with p53, which is elevated approximately fivefold relative to the level at 32°C.

This explanation for the ability of PAb421 and 4B2 only being able to co-precipitate p53 and MDM2 together at 32°C, but not at 37°C is consistent with difference in level of MDM2 at the two temperatures, and also with the published observations that *mdm2* expression is dependent on the 'wild-type' form of p53 predominantly present at 32°C (Barak *et al.*, 1993; Wu *et al.*, 1993).

The failure of Bp53-19 to co-immunoprecipitate MDM2 or detect the p53-MDM2 complex at 32°C is unexpected for two reasons. Firstly, the two-site assay suggests there is MDM2 protein in excess, which is able to form complexes with p53 as detected by the capturing antibodies PAb421 and 4B2. Secondly, the two-site immunoassay at 37°C suggests that Bp53-19 is almost as efficient as PAb421 at recognizing p53 in the cell extracts. The simplest interpretation for this observation is that BP53-19 recognizes the same region on p53 that MDM2 binds to.

Preliminary evidence from work in this laboratory and elsewhere has shown that Bp53-19 (Stephen *et al.*, manuscript in preparation) and MDM2 interact with amino terminus of p53 (Oliner *et al.*, 1993). Following on from epitope mapping projects in this laboratory, a complete peptide library of the human p53 protein, and a partial peptide library of the mouse p53 protein, were available to identify the region to which MDM2 binds. These libraries consisted of 15 amino acid long sections of the p53 primary amino acid sequence, that consecutively overlapped by five amino acids, and were each attached to biotin via a four amino acid long spacer. By immobilizing the biotinylated peptides on streptavidin-coated ELISA plates the MDM2 binding site on p53 could be quickly identified provided it is encompassed within a stretch of 15 amino acids or less. Extract containing MDM2 was added to an ELISA plate with the peptide library bound to it, and the bound MDM2 protein was later detected using monoclonal antibody 4B2 and the standard ELISA assay. Several sources of recombinant MDM2 protein were used to challenge the p53 library, these included crude extracts and partially purified preparations of human and mouse MDM2 expressed in *E. coli* and also mouse MDM2 expressed in insect cells. All forms of MDM2 that we tested identified the same peptides in the p53 library. The results using the mouse MDM2 expressed in insect cells are shown in Figure 3a and b, and are presented alongside the ELISA readings for extract of insect cells alone not expressing mouse MDM2. The specificity is remarkable, suggesting a strong interaction between MDM2 and p53-derived

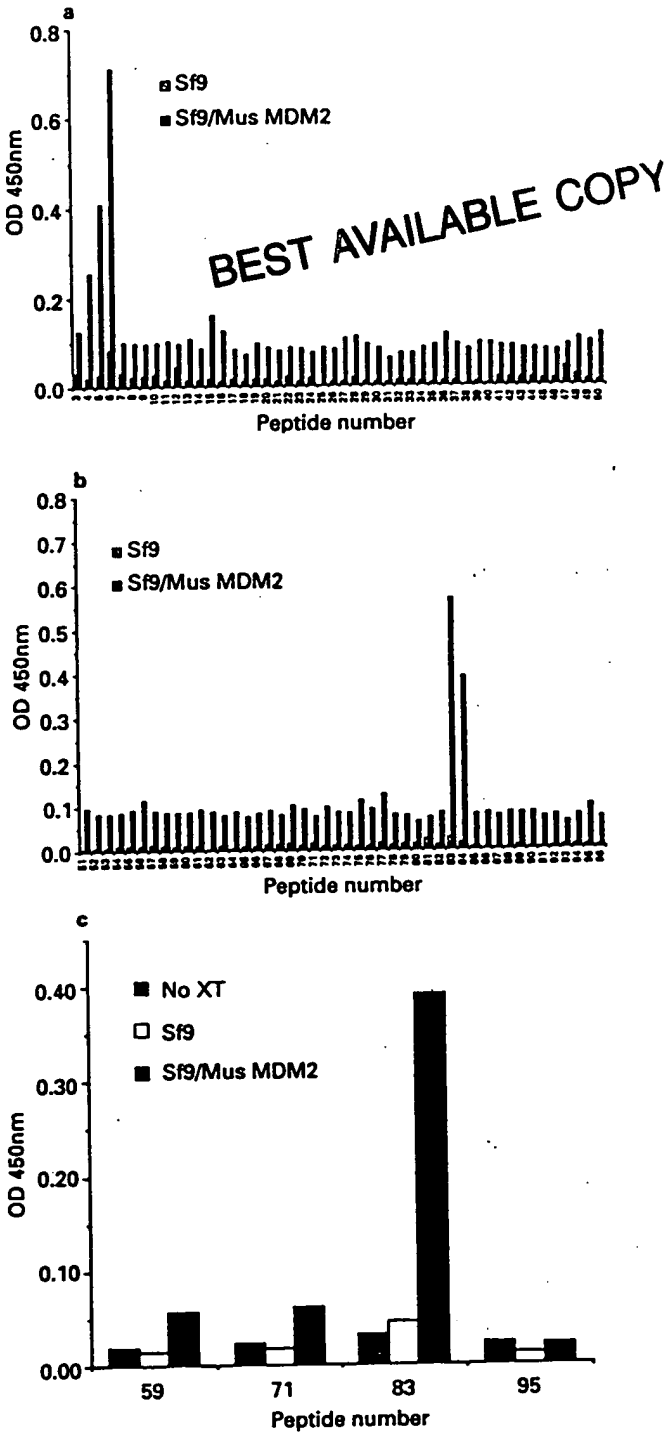

Figure 3 Identification of the MDM2 binding site on human and mouse p53. In (a) is shown the results for peptides numbers 3–50, representing the N-terminal to mid region of human p53 and in (b) the remainder of the human p53 amino acid sequence and the N-terminus of mouse p53. The human p53 sequence starts at peptide number 3 and ends at peptide 79, and each peptide consists of 15 amino acids, with the last five amino acids being present in the next peptide along. The mouse p53 sequence is partial and consists of the N-terminal sequence from amino acid 1–92, again each overlapping the next and previous peptide by five amino acids. The peptide library was challenged with insect cell extract alone, Sf9, and insect cell extract expressing mouse MDM2, Sf9 Mus MDM2. Binding of MDM2 to the peptides was determined by an ELISA assay using monoclonal antibody 4B2, and then detecting bound antibody with rabbit anti-mouse Ig conjugated horse radish peroxidase and TMB substrate. In (c) is shown the results from a control experiment using peptides 59, 71, 83 and 95, as used in (b) but conducted in the presence or absence of extract (XT) to verify the specificity of the detecting antibody, 4B2

peptides. From the controls shown in Figure 3c, it can be seen that the binding is only observed in the presence of extract expressing MDM2, and is not due to the antibody recognizing the peptide alone, moreover, identical results were obtained using SMP14 as the primary detecting antibody (data not shown).

The four peptides that bind MDM2 are shown in Figure 4. Peptides 5 and 6 identify a site at the N-terminus of human p53, whereas peptides 83 and 84 identify the corresponding region in the N-terminus of mouse p53. Collectively, these four peptides define the consensus MDM2 binding site on p53 to be -QETFSD/GLWKL, the aspartate to glycine being the only amino acid difference between the human and mouse sequence. The peptides involved in binding MDM2 are also those recognized by the p53 antibodies DO-1 and Bp53-19 (Stephen *et al.*, manuscript in preparation).

To define key residues on p53 that are involved in the interaction with MDM2 a form of the consensus binding site sequence -QETFSDLWKL- was modified by substituting alanine at each position in the sequence

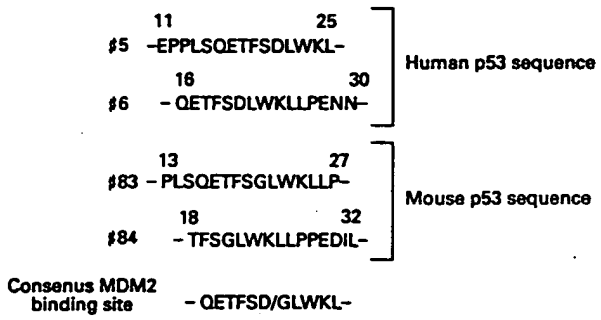

Figure 4 This figure shows the peptide sequences to which MDM2 bound, and defines the consensus MDM2 binding site on human and mouse p53

and determining what effect this has on the binding of MDM2 from the insect cell extract expressing MDM2. This experiment was conducted in concert with examining the effect on binding of the antibodies DO-1 and Bp53-19. The results are presented in Figure 5. With regard to MDM2 binding all alanine substitutions in the consensus binding site reduce the level of binding as measured by ELISA, however, the key residues would appear to be TFSDLW as substitutions in these positions reduce the amount of MDM2 binding to less than 15% of that seen with the unchanged consensus sequence. Interestingly, a higher level of binding of MDM2 is observed to the smaller consensus peptide rather than to peptide 6 (QETFSDLWKLLPENN) of the p53 peptide library reaffirming the definition of the binding site. In the case of monoclonal antibody DO-1 binding to the consensus sequence the key residues are ETFSDLWK, with D and K being the most crucial. The importance of the asparate residue to the DO-1 epitope is consistent with the report that DO-1 only recognizes human p53 and not mouse p53 (Vojtesek *et al.*, 1992), – the only difference being an aspartate to glycine change. While this difference has a critical affect on DO-1 binding it does not grossly affect the interaction of MDM2 with the protein or peptides. However substitution of alanine for aspartate at this position blocks binding of all three protein ligands. The ability of MDM2 to distinguish alanine from either glycine or aspartate at this position may imply that the polar environment of this region of the binding site is critical for the interaction. It has also been established from phage display libraries that the epitope of DO-1 is FSDLWKL (Stephen *et al.*, manuscript in preparation), which is in agreement with our observations on key residues. For the antibody Bp53-19 the alanine substitution series identifies the key residues to be F-DLW, with the latter three residues being the most crucial, and is

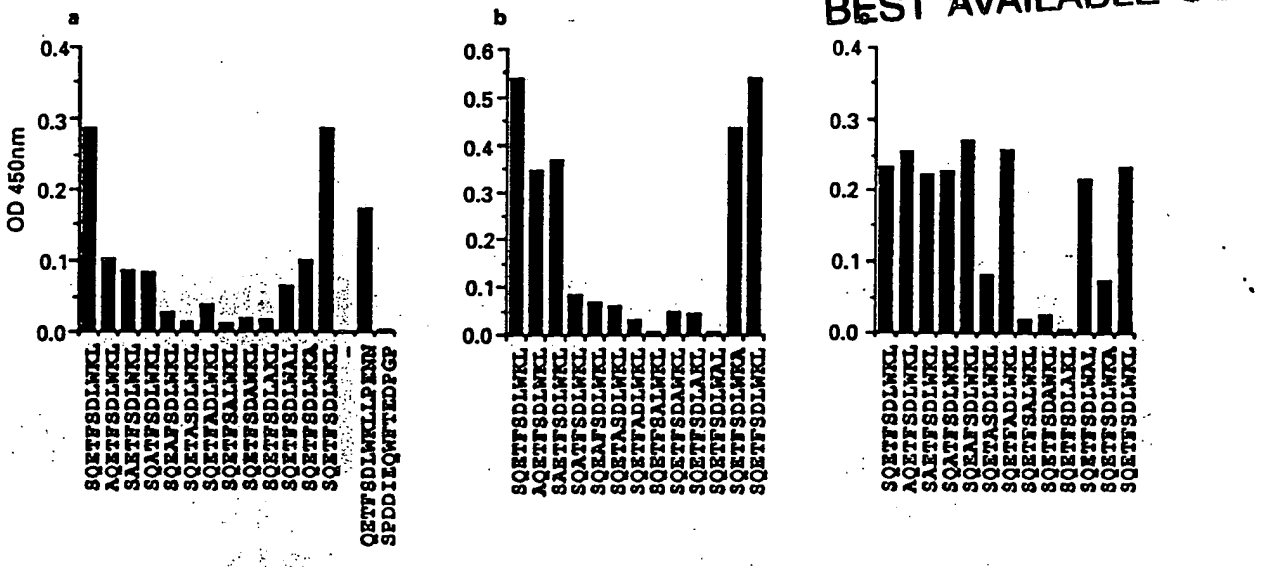

Figure 5 Identification of the key residues required for binding of MDM2 (a), antibody DO-1 (b) and antibody Bp53-19 (c), by sequential alanine substitution at the MDM2 binding site. The amino acid sequences are as stated. In (a) the sequence QETFSDLWKLLPENN represents the sequence of peptide 6 from Figure 3 and SPDDIEQWFTEDPGP is an irrelevant peptide control. Formally the first serine residue on the stated peptide sequence is part of the spacer coupling the consensus peptide to biotin, since serine also precedes the consensus p53 sequence this residue was also substituted with alanine.

similar to the requirements for MDM2 binding to the consensus binding site. Not surprisingly, the pre-binding of antibody Bp53-19 to the QETFSDLWKL biotinylated peptide blocked binding of MDM2 to the peptide when added later (data not shown).

Discussi n

In the work presented here we have re-examined in a quantitative manner the formation of a p53-MDM2 complex in Clone 6 cells at 32°C and 37°C. Our results confirm previous immunoprecipitation observations that the level of MDM2 at the lower temperature is significantly elevated, approximately 10–30 fold greater than that at 37°C, at which temperature MDM2 is only just detectable. Consequently, the p53-MDM2 complex is readily observed at 32°C and not at 37°C. The level of p53 also varies at the two different temperatures. However, the p53 level is elevated approximately fivefold at 37°C as compared with that at 32°C, the opposite behaviour to that of MDM2. Accordingly, the difference in the levels of p53 and MDM2 are likely to have alternative explanations. In the case of MDM2, other groups have established that the increase of MDM2 at 32°C is due to increased transcription of MDM2 due to a conformational change in p53 to a presumed transcriptionally active form (Barak *et al.*, 1993; Wu *et al.*, 1993). The same explanation does not apply for p53 even though wild type p53 is implicated in being involved in its own expression (Deffie *et al.*, 1993), and is probably explained by the increased half life of the mutant conformation of p53 at 37°C (Gannon & Lane, 1991). Our data using both direct observation of the p53-MDM2 complex by ELISA and immunoprecipitation combined with the indirect inference of the loss of the Bp53-19 epitope suggest that nearly all p53 molecules are complexed to excess MDM2 protein in Clone 6 cells at 32°C. This is not consistent with the powerful p53-dependent transcriptional response seen in these cells at this temperature and suggests either that complexing to MDM2 is unable to completely inactivate p53 *in vivo* or that small amounts of 'free' p53 may be very active. The complex between p53 and MDM2 may be regulated in cells to release functional p53 at the individual cell level, perhaps as a cell cycle dependent response. Others have reported that only a minority of p53 is bound to MDM2 in the Clone 6 cell line (Barak *et al.*, 1993), based on sequential immunoprecipitation with the same reagents used in the ELISA assay in Figure 2, it is not clear why there is such a disparity between the results obtained with the two different methods.

The major finding of this report is identification of the minimal MDM2 binding site to be TFSD/GLW. This site is in a location broadly reported by other groups to be the MDM2 binding domain of p53, specifically aa 1–41 and 13–57 (Olin r *et al.*, 1993), aa 1–52 (Chen *et al.*, 1993) and aa 1–159 (Brown *et al.*, 1993). Notably, a construct generated by Oliner and co-workers (1993) encompassing aa 13–41 of p53 was not sufficient for MDM2 binding in a three hybrid protein system, and differs from our observations. The disparity might be explained by the close proximity of the fusion protein sequence to the TFSD/GLW sequence at aa 18–23 as our data show that flanking sequences do contribute in a minor way to MDM2 binding. The TFSD/GLW sequence is very close to the transactivation domain aa 20–42 (Unger *et al.*, 1992), and as shown by others the binding of MDM2 to this site interferes with the transcriptional activity of p53 (Oliner *et al.*, 1993). While substitution analysis of the MDM2 binding site on p53 identified the TFSD/GLW sequence to be the key region required for MDM2 to bind p53, other residues flanking this site also contribute in a minor way to MDM2 binding, but clearly the TFSD/GLW sequence is a minimal target for agents that might disrupt complex formation without affecting the transactivation activity (for which as yet the key residues are undetermined). The first three residues TFS are part of the conserved box I as defined by Soussi *et al.* (1990), and the latter three D/GLW are outside but are also part of a region of p53 that shows few sequence changes from *Xenopus* to man. Following data base searches using this consensus motif, we have identified a putative MDM2 binding site in another oncogene and this is now being functionally tested.

The corresponding binding site on MDM2 for p53 has variously been reported to be between aa 1–121, 19–102 (Chen *et al.*, 1993) together with aa 102–294 or 249–491, and also 1–221 (Brown *et al.*, 1993). Notably, a monoclonal antibody against the N-terminal region of human MDM2, 3G5 (maps at aa 59–89) is able to immunoprecipitate MDM2 but not co-immunoprecipitate p53 (Chen *et al.*, 1993), an analogous observation to our findings with antibody Bp53-19.

The binding of MDM2 to p53 peptides has obvious parallels to a similar study that used small peptides to identify the binding sites of Adenovirus E1A and human papilloma virus E7 for a range of proteins including retinoblastoma protein, p107, cyclin A and p130 (Dyson *et al.*, 1992a,b). The MDM2 binding site on p53 appears to be a single domain rather than two domains, as in the case of E1A and E7. The MDM2 binding site on p53 overlaps precisely with a highly immunogenic epitope on the protein; many independently isolated monoclonal antibodies to p53 recognise the site, and antibodies to it are present in the sera of cancer patients (Schlichtholtz *et al.*, 1993). This suggests that it has an exposed and defined structure. It is possible that the amino acid sequence of the complementarity determining regions of these antibodies will show homology to the p53 binding site of MDM2. It also suggests that anti-p53 antibodies used to examine p53 levels where high levels of MDM2 are present must be chosen with care. Binding of MDM2 to this site may be regulated by phosphorylation since an alanine substitution at serine-20 abolishes binding, thereby identifying it as a key residue, and there is known to be a DNA-dependent kinase site at serine 20 (Lees-Miller *et al.*, 1990) and other phosphorylation sites at serine 6, 9 and 15 on p53 (Samad *et al.*, 1986; Meek & Eckhardt, 1988). This issue is presently being addressed. The therapeutic opportunity is to develop small molecules based on this binding site that might act to free functional wild type p53 from the excess levels of MDM2 protein present in some human tumours. Currently, we are investigating this opportunity using synthetic combinatorial peptide libraries (Houghten *et al.*, 1991).

Materials and methods

Cell cultures

Clone 6 cells (Michalovitz *et al.*, 1990) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS at either 32 or 37°C. The *Spodoptera frugiperda* cell line, Sf9, was grown at 27°C in ExCell 400 medium (J.R. H. Biosciences, Sera-Lab, UK) supplemented with 5% FCS and glutamine.

Expression of mouse mdm2 *in insect cells*

The mouse *mdm2* gene was obtained from a mouse prostate cell line (Lu *et al.*, 1992) by polymerase chain reaction and then cloned into a *Spodoptera frugiperda* expression vector pVL1393 using standard DNA and baculovirus expression techniques. An expression clone was identified by the production of a 90–95 kDa protein that was recognized by anti-MDM2 antibodies.

Antibodies

p53 protein was detected using the polyclonal serum CM1 (Midgley *et al.*, 1992) or monoclonal antibodies PAb421 (Harlow *et al.*, 1981), DO-1 (Vojtesek *et al.*, 1992) and Bp53-19 (Bartek *et al.*, 1993). MDM2 was detected using rabbit anti-MDM2 polyclonal sera (Barak *et al.*, 1993) or m noclonal antibody 4B2 (Chen *et al.*, 1993) and SMP14 (a previously unreported monoclonal antibody raised by us against a peptide, CSRPSTSSRRRAISE, containing part f the human MDM2 sequence from aa 154 to 167 (Oliner *et al.*, 1992). The first cysteine is not part of the MDM2 sequence but was added to provide an extra coupling option. An antibody, PAb419, raised against SV40 large T antigen (Harlow *et al.*, 1981) was used as an irrelevant control for immunoprecipitations.

Immunoprecipitations

Cells were lysed in ice-cold NET buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP40) containing 1 mM phenylmethylsulphonyl fluoride, for 30 min at 4°C. Debris was removed from the cell extract by centrifugation at 14 000 r.p.m. in a refrigerated Eppendorf centrifuge. The immunoprecipitation procedure was essentially as previously described (Gannon *et al.*, 1990) using 1 μg of purified mouse monoclonal antibody, and Protein G Sepharose beads (Pharmacia) for both pre-absorption of the cell extracts and subsequent isolation of the antibody-protein complex.

Screening of p53 peptide library

Peptide libraries of the entire human p53 protein and a partial N-terminal region of the mouse p53 protein was btained from Chiron Mimotopes P/L (Victoria, Australia). The libraries were in the form of 15 mer peptides linked to biotin via an additional peptide spacer region of serine-glycine-serine-glycine, and each peptide shared a five amino acid overlap with the previous peptide in the primary sequence. The libraries were synthesised in a ninety six well format with the first two wells being used for quality control purp ses. Accordingly, the human p53 peptide library starts at well number three and contains the amino acid sequence (SGSG)-^{1}MEEPQSDPSVEPPLS^{15}, the sequence in well 4 is (SGSG)-^{6}SDPSVEPPLSQETFS^{20}, and so on until ending at well 79 with the sequence (SGSG)-^{378}RHKKLMFKTEGPDSD^{393}. The mouse library starts with the sequence (SGSG)-$^{-3}$MTA^{1}MEESQSDISLEL^{12} at well 80, followed by (SGSG)-^{3}ESQSDISLELPLSQE^{17} and continues as far as (SGSG)-^{78}APAPATPWPLSSFVP^{92} in well 96.

ELISA plates were coated with 100 μl of 5 μg ml^{-1} streptavidin (Vector labs) per well and incubated overnight at 37°C and then blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at room temperature. The stock biotinylated peptides were diluted to 5 μg ml^{-1} in PBS containing 0.1% BSA and 50 μl of each were plated into designated wells and then incubated at room temperature for 1 h. The plates were washed four times with PBS containing 0.1% Tween 20 before addition of the cell extract (50 μl of 1–4 mg ml^{-1} per well) or purified protein. The plates were incubated at 4°C for 2–3 h, before washing four times with PBS containing 0.1% Tween 20 to remove unbound protein. In the case of cell extracts, bound protein was detected with the appropriate primary antibody at 1–3 μg ml^{-1}, and followed by an anti-mouse horseradish peroxidase conjugate and 3'3'4'4'-tetramethyl benzidine (TMB) substrate as in the standard ELISA assay (Harlow & Lane, 1988). Plates were read at OD 450 nm using a Dynatech 500 plate reader.

ELISA assay

The levels of p53, MDM2, and complexes thereof were determined by a two site immunoassay using stated antibodies. Mouse monoclonal antibodies were used as the solid phase by incubating Falcon microtitre dish wells with 50 μl of a 30 μg ml^{-1} solution of purified antibody overnight at 4°C. The plates were blocked with 2% bovine serum albumin in PBS for 2 h at room temperature and washed with PBS. Cell extracts were prepared as described for immunoprecipitations and then serially twofold diluted before adding 50 μl per well and incubating at 4°C for 2 h. The plates were then washed with 0.1% NP-40 in PBS, before addition of 50 μl of detecting polyclonal antisera at 1/1000 dilution. The plates were washed again with 0.1% NP-40 in PBS and 50 μl of 1/1000 dilution peroxidase-conjugated swine anti-rabbit Ig serum (DAKO) was added for 2 h, then visualised by the TMB reaction.

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Biochemistry

DNA-dependent protein kinase activity is absent in *xrs-6* cells: Implications for site-specific recombination and DNA double-strand break repair

(antigen Ku)

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ABSTRACT DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase composed of a catalytic subunit called p350 and a DNA binding component termed Ku. Ku consists of two tightly associated polypeptides of approximately 70 kDa and 80 kDa (Ku80). An intriguing feature of DNA-PK is that it binds to DNA ends and other discontinuities in DNA and requires these structures for its activation. This suggests that DNA-PK may function in DNA repair and/or recombination. Consistent with this, Ku DNA binding activity was shown recently to be absent in extracts of hamster *xrs-6* cells, which are defective in DNA double-strand (ds) break repair and V(D)J recombination. Furthermore, *xrs-6* cells are complemented by expression of the Ku80 cDNA. To date, DNA-PK activity has been demonstrated unequivocally only in extracts of primate cells. Here, we describe an assay that can detect DNA-PK activity in extracts of mouse, hamster, *Xenopus*, and *Drosophila* cells. Using this assay, we find that *xrs-6* cells completely lack DNA-PK activity. By contrast, *xrs-6* derivatives complemented by human chromosome fragments bearing the Ku80 gene have restored both the DNA end binding and kinase activities associated with DNA-PK. Finally, we show that *xrs-6* extracts are complemented biochemically by purified Ku. Our findings indicate that the *xrs-6* defects are direct consequences of the mutation in Ku80 and implicate DNA-PK in recombination and DNA repair processes.

A protein kinase that appears to be well suited to controlling transcription and other nuclear events is DNA-dependent protein kinase (DNA-PK), an abundant enzyme in human cells that is located primarily within the nucleus (1–5). An important feature of DNA-PK is that it interacts with DNA and must be DNA-bound to be active. We and others (6–8) have shown that DNA-PK consists of two components. One, the catalytic component, is a protein of ~350 kDa (p350). On its own, however, p350 is inactive and requires the second DNA-PK component to target it to DNA and trigger its kinase activity. This second component is human autoimmune antigen Ku, which is composed of two tightly associated polypeptides of approximately 70 and 80 kDa (Ku70 and Ku80, respectively).

Although *in vivo* targets for DNA-PK have not yet been defined unequivocally, many transcription factors, including Sp1, c-Jun, c-Fos, p53, and CTF1/NF-I are phosphorylated by this enzym *in vitro* (2, 3, 7, 9–11). One function of DNA-PK may therefore be to control gene expression. However, since transcription factors can also influence nuclear events such as DNA replication, recombination, and DNA repair (12–15), these processes might also be affected by DNA-PK-mediated phosphorylation.

We and others (16–18) have shown recently that Ku DNA binding activity is absent in the Chinese hamster ovary cell line *xrs-6*, which is impaired in DNA double-strand break repair and V(D)J recombination (19–21). The Ku80 gene maps to the region of human chromosome 2 containing the gene *XRCC5*, which complements the *xrs-6* mutation (22–24). Furtherm re, we have demonstrated that *xrs-6* cells can be complemented by expression of the Ku80 cDNA, indicating that *XRCC5* encodes Ku80 (18). An involvement of DNA-PK in DNA double-strand break repair would be consistent with the fact that this enzym requires DNA termini for its activation *in vitro* (1, 2, 4, 8, 25, 26). We therefore wished to determine whether the kinase activity associated with DNA-PK is also impaired in *xrs-6* cells. Here, we develop an assay scheme that can detect DNA-PK activity in extracts of rodent, *Xenopus*, and *Drosophila* cells and use this to determine DNA-PK levels in *xrs-6* cell extracts. The results of these studies suggest that the DNA double-strand break repair and recombination defects of *xrs-6* cells may be due not only to the loss of Ku DNA end binding activity but also to the concomitant loss of DNA-PK catalytic function.

MATERIALS AND METHODS

Cells, Extract Preparation, and Protein Purification. Mammalian cells, *Xenopus laevis* cell line XL-177, and their propagation were as described (22, 27). Extracts were prepared by a modification of the method of Scholer *et al.* (28): frozen cell pellets (0.5–3 × 10^7 cells) were resuspended in 100 μl of extraction buffer [50 mM NaF, 20 mM Hepes (pH 7.8), 450 mM NaCl, 25% (vol/vol) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), protease inhibitor (0.5 μg/ml), trypsin inhibitor (1.0 μg/ml), aprotinin (0.5 μg/ml), bestatin (40 μg/ml)] and then frozen on dry ice and thawed at 30°C three times. After microcentrifugation for 7 min at 4°C, supernatants were stored at −70°C. Ku was purified essentially as described (6).

DNA-PK Assays. Standard peptide phosphorylation assays were as described (4). DNA-PK "pulldown" kinase assays were conducted as follows. Extract was incubated with 20 μl of preswollen double-stranded DNA (dsDNA)-cellulose (Sigma) in a total volume of 50 μl of Z'0.05 (25 mM Hepes/KOH at pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol). The dsDNA-cellulose was then wash d twice with 1 ml of Z'0.05 and resuspended in 50 μl of Z'0.05. Samples were divided into two r thre aliquots, 0.5 μl of [γ-^{32}P]ATP (300 Ci/mmol; 1 Ci = 37 GBq) was

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Abbreviations: DNA-PK, DNA-dependent protein kinase; dsDNA, double-stranded DNA; wt, wild type; WCE, whole cell extract.

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added, and kinase assays were conducted in the presence or absence of 4 nmol of peptide (0.2 mM). Reactions were then stopped and analyzed by spotting ont phosphocellulose paper, washing, and subjecting to liquid scintillation counting as described (4). The sequences of wild-type (wt) and mutant p53 peptides are EPPLSQEAFADLLKK and EPPLSEQAFADLLKK, respectively. All assays were performed multiple times with at least two different extract preparations. Reproducibility of DNA-PK pulldown peptide assays (cpm incorporated for a given extract) is generally less than ±10%.

RESULTS

Standard DNA-PK Assays Do Not Detect DNA-PK in Rodent Cell Extracts. As Ku has been reported to be a component of DNA-PK, we wished to test whether DNA-PK activity is defective in *xrs-6* cell extracts. The most sensitive DNA-PK assay hitherto described uses a synthetic peptide derived from the N-terminal transcriptional activation region of murine p53 (4). In this assay, the peptide is incubated with DNA-PK and [γ-^{32}P]ATP, adsorbed onto derivatized paper, and peptide phosphorylation is determined by liquid scintillation counting after washing away unbound material. Generally, reactions are performed both in the absence and presence of DNA. Since DNA-PK appears to be the only DNA-activated kinase in mammalian cells (2, 4), the difference of these two values provides a quantitative estimate of DNA-PK activity. As shown in Fig. 1, using this method, DNA-activated peptide phosphorylation is detected readily using whole cell extract (WCE) from human 1BR cells. This kinase activity is indeed DNA-PK, because no DNA-activated phosphorylation is observed with a mutated peptide (MUT PEP) in which the DNA-PK phosphorylation site *Ser*-Gln is mutated to the sequence Ser-Glu, which is not recognized by DNA-PK (4, 11). Consistent with previous reports (4), this standard assay does not detect DNA-PK activity in extracts of hamster or mouse cells (Fig. 1).

Development of a DNA-PK Pulldown Peptide Assay. We considered it likely that DNA-PK would be present in rodents, because Ku homologs exist in mouse (29, 30) and Western blotting suggests that p350 is present in both murine and hamster cells (T.M.G. and S.P.J., unpublished data). Possible explanations for our inability to observe DNA-PK in rodent extracts were that its activity might be too low for detection or that its actions might be reversed by phosphatases. Thus, we reason d that fractionation of rodent extracts might reveal DNA-PK activity. W therefore devised a microscale pulld wn purification scheme for DNA-PK that takes advantage of the fact that this enzyme binds to dsDNA, whereas most protein phosphatases and kinases do not. In this assay, crude cell extract is incubated with dsDNA-cellulose, and then unbound material is removed by repeated washing. Because DNA-PK retains its activity when bound to DNA-cellulose, the immobilized enzyme is assayed simply by adding buffer, p53 peptide, and [γ-^{32}P]ATP. High levels of p53 peptide kinase activity are recovered from human cell extracts using this approach (Fig. 2*A*). Addition of more extract yields higher levels of recovered activity, and an approximately linear relationship exists between the amount of extract and kinase levels over a wide range of extract concentrations. Furthermore, the pulldown assay is effective both with crude nuclear extracts (Fig. 2*A*) and WCEs (Fig. 2*B*). Since the recovered kinase is already DNA-bound, assays cannot be performed in the absence of DNA. Nevertheless, we conclude that the activity recovered is indeed

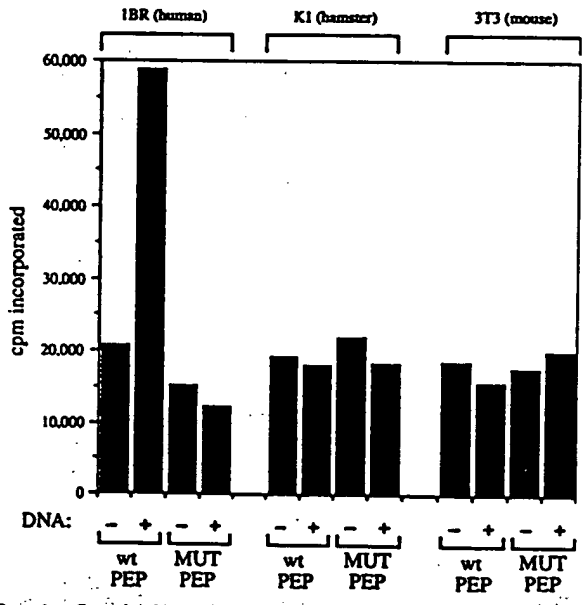

FIG. 1. Standard peptide assays detect DNA-PK in extracts of human but not rodent cells. WCE derived from human 1BR, hamster K1, or mouse 3T3 (10 μg, 40 μg, and 40 μg, respectively) cells was incubated with wt peptide (wt PEP) or mutant p53 peptide (MUT PEP) as indicated, in the presence of [γ-^{32}P]ATP, and the amount of peptide phosphorylation was determined. Reactions were conducted either in the absence (−) or presence (+) of linearized plasmid DNA. cpm incorporated into peptide for a typical assay are plotted.

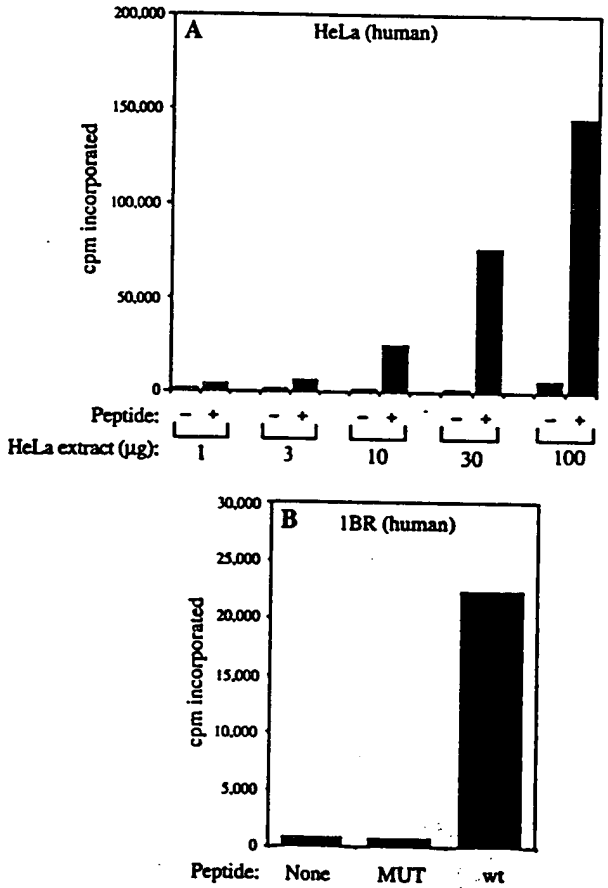

FIG. 2. DNA-PK pulldown peptide assay. (*A*) Titration of HeLa extract. Increasing amounts of HeLa nuclear extract (as indicated) were incubated with dsDNA-cellulose, and bound material was collected and washed. This was then incubated with [γ-^{32}P]ATP, either in the absence (−) or presence (+) of wt p53 peptide, and the amount of radiolabel incorporated was determined. (*B*) The kinase precipitated in the pulldown assay displays DNA-PK substrate specificity. Pulldown experiments were conducted using 25 μg of WCE from human 1BR cells, and phosphorylation reactions were conducted either in the presence of mutant (MUT) or wt p53 peptide or in the absence of peptide (none).

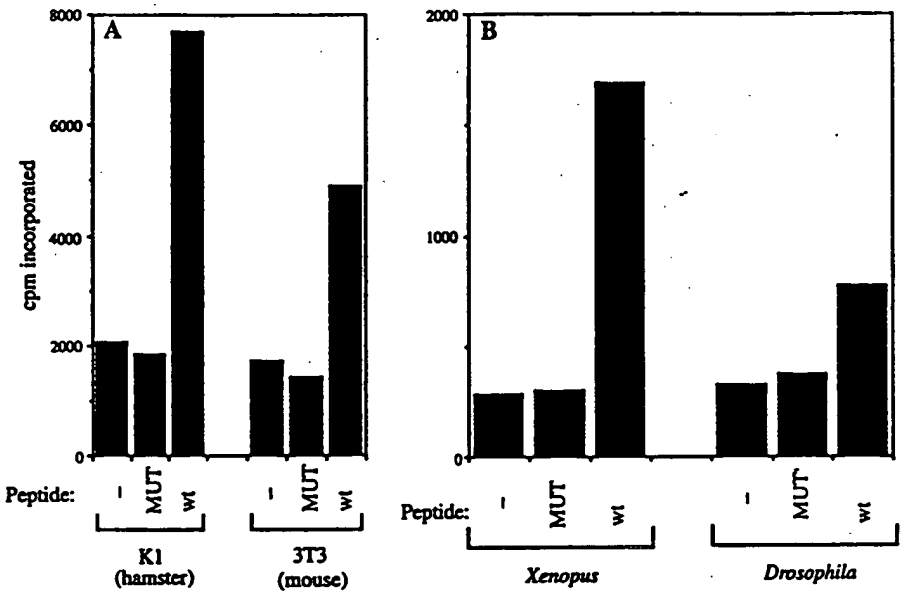

FIG. 3. The DNA-PK pulldown peptide assay detects DNA-PK in rodent, *Xenopus*, and *Drosophila* extracts. (*A*) WCE (150 μg) from hamster K1 or mouse 3T3 cells was used in a DNA-PK pulldown peptide phosphorylation assay with wt or mutant (MUT) p53 peptide or in the absence f peptide (−). (*B*) WCE (150 μg) derived from *X. laevis* cell line XL-177 or *Drosophila* embryos was used in a DNA-PK pulldown peptide phosphorylation assay with wt or mutant p53 peptide or in the absence of peptide (−).

DNA-PK, because the mutant p53 peptide is not phosphorylated significantly (Fig. 2*B*).

Detection of DNA-PK Activity in Extracts of Hamster, Mouse, *Xenopus*, and *Drosophila* Cells. Having established a more sensitive assay for DNA-PK, we next analyzed extracts of rodent cells. As shown in Fig. 3*A*, p53 peptide kinase activity is detected reproducibly in both hamster and mouse WCEs. Furthermore, kinase activity is observed with the wt but not the mutant p53 peptide, suggesting strongly that it indeed corresponds to rodent DNA-PK. Through performing titration experiments (data not shown), we estimate that mouse and hamster cell extracts contain ~50-fold less DNA-PK than extracts of human HeLa and 1BR cells. Interestingly, DNA-PK activity is also detected in extracts of *X. laevis* XL-177 cells and of *Drosophila melanogaster* embryos (Fig. 3*B*), suggesting that DNA-PK has an important and evolutionarily conserved function throughout the eukaryotic kingdom.

DNA-PK Activity Is Absent in Extracts of *xrs-6* Cells. Since *xrs-6* cells lack Ku DNA binding activity, we used the pulldown assay to test extracts of these cells for DNA-PK. Thus, we discovered that, unlike the wt hamster cell line K1, *xrs-6* cells contain no DNA-PK activity (Fig. 4*A*). This deficiency appears to be linked to the DNA damage and recombination defects of *xrs-6* cells because DNA-PK activity is also undetectable in extracts of the independently derived hamster lung cell line XR-V15B, which falls into the same complementation group as *xrs-6* (Fig. 4*B*).

***xrs-6* Cells Complemented by Human DNA Fragments Have Restored DNA-PK Levels.** We have recently shown that the Ku80 gene corresponds to the human *XRCC5* gene (18). Th

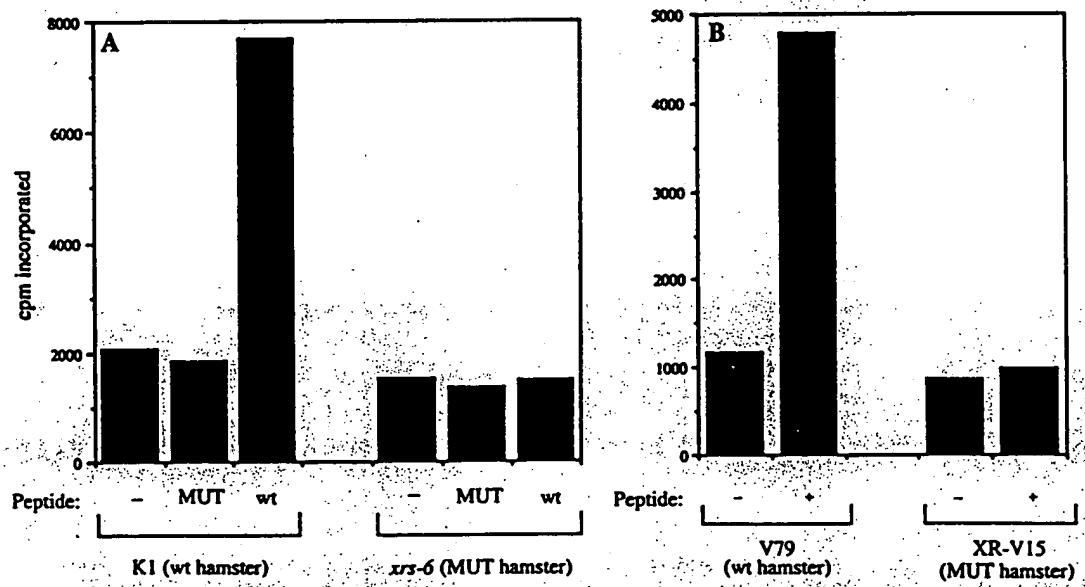

FIG. 4. *xrs-6* and XR-V15 cells lack DNA-PK. (*A*) K1 or *xrs-6* extract (150 μg) was analyzed by the DNA-PK pulldown peptide assay with wt or mutant (MUT) p53 peptide or in the absence of peptide (−). (*B*) DNA-PK pulldown peptide assays were performed using V79 or XR-V15 cell extracts in the absence (−) or presence (+) of wt p53 peptide substrate.

Ku80 gene maps to the human chromosome region 2q33-35 (24), and we have generated a series of hamster–human cell hybrids derived from *xrs-6* cells that c ntain different portions of human DNA from this region (22, 23). Some hybrids, such as H22 and D2-X-38, contain th Ku80 gene and complement th *xrs-6* mutation, wh reas others such as 38D do not contain an intact Ku80 gene and do not complement the mutation (18, 23). When extracts from these clones were used in DNA-PK pulldown assays, we found that complementing hybrids H22 and D2-X-38 have DNA-PK activity, whereas noncomplementing hybrid 38D lacks detectable DNA-PK (Fig. 5*A*). Kinase activity is consistently lower in hybrid D2-X-38 compared to hybrid H22, which correlates with the intermediate radiosensitivity of D2-X-38, which we have attributed to instability of the human DNA fragment in this line (18). These results indicate that the defect in Ku80 in *xrs-6* cells also results in a loss of DNA-PK activity.

Biochemical Complementation of *xrs-6* Extracts with Purified Ku. If the DNA-PK defect of *xrs-6* extracts is due solely to a lack of Ku, then the addition of purified Ku should restore DNA-PK activity. Previously, we have shown that p350 cannot bind DNA in the absence of Ku (8) and therefore anticipated that Ku w uld restore kinase activity only if added prior to the dsDNA-cellulose pulldown step. Consistent with this, when Ku is added to dsDNA-cellulose that has been preincubated with *xrs-6* extracts, no complementation is achieved (data not shown). By contrast, when a purified preparation of Ku is mixed with the *xrs-6* extract before adding the dsDNA-cellulose, DNA-PK activity is obtained (Fig. 5*B*). We thus conclude that *xrs-6* cells contain active p350 and that the DNA-PK defect is due to a specific deficiency in Ku.

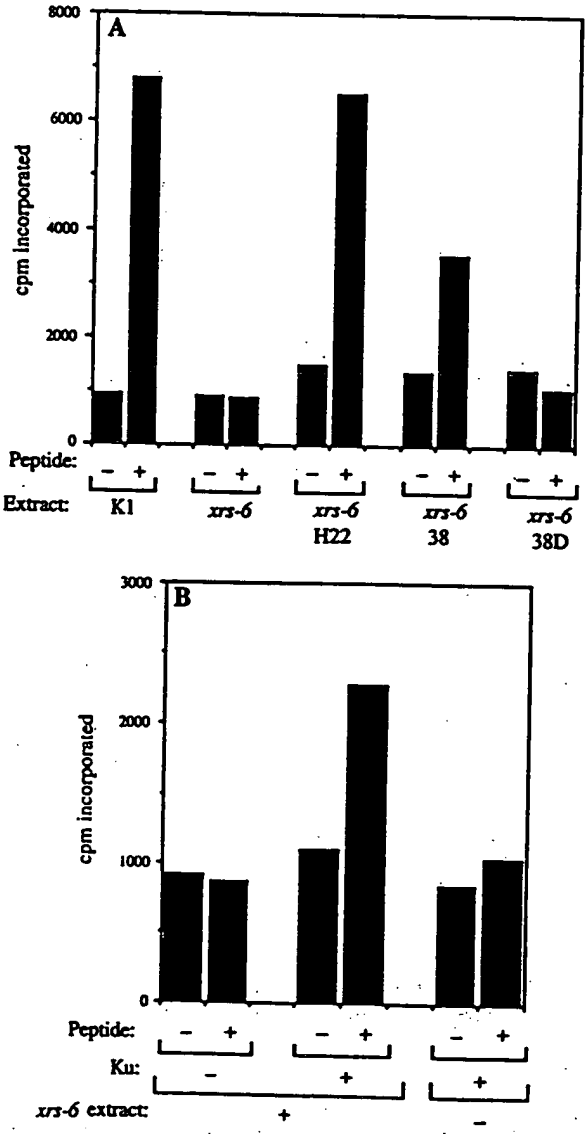

FIG. 5. (*A*) DNA-PK activity correlates with radiation resistance. Extracts (150 μg), as indicated, were analyzed by the DNA-PK pulldown peptide assay in the absence (−) or presence (+) of wt p53 peptide. Cell lines employed are described in the text. (*B*) Biochemical complementation of the *xrs-6* DNA-PK defect. *xrs-6* extract alone (150 μg), *xrs-6* extract (150 μg) in the presence of 200 ng of purified Ku, or 200 ng of Ku alone was analyzed by the DNA-PK pulldown peptide assay.

DISCUSSION

In this paper, we describe an assay scheme for DNA-PK that employs a microscale DNA-PK pulldown purification on dsDNA-cellulose as its first step. This assay is much more sensitive than standard DNA-PK assays for several reasons. First, the pulldown step can concentrate DNA-PK from dilute samples. Second, because most kinases do not bind to dsDNA-cellulose significantly, background phosphorylation by other kinases is reduced greatly. Third, the pulldown step removes endogenous kinase substrates that contribute to the high levels of phosphorylation in standard DNA-PK assays in the absence of peptide. Finally, protein phosphatases that reverse phosphorylation by DNA-PK are removed by the pulldown step.

Using this technique, we have shown that DNA-PK exists in hamster, mouse, *Xenopus*, and *Drosophila* cells. This is consistent with the fact that Ku homologs have been identified in mouse and *Drosophila* (29–31) and suggests strongly that DNA-PK may play a role in DNA repair and site-specific recombination in diverse eukaryotes. Indeed, the recent identification of a Ku-like activity in *Saccharomyces cerevisiae* (32) suggests that Ku/DNA-PK is ubiquitous in the eukaryotic kingdom. Interestingly, we find that primate cells have 10- to 50-fold more DNA-PK activity than other species that we have examined. Although the reason for this is unclear at present, it is tempting to speculate that primate cells may be particularly effective at recognizing DNA double-strand breaks or performing certain types of site-specific recombination. Wh n w examined extracts of *xrs-6* and XR-V15B cells, we found them to be devoid of DNA-PK activity. Since this deficiency is complemented by the addition of purified Ku, this reinforces our prior conclusion that the primary defect of *xrs-6* cells is the DNA end-binding activity associated with Ku. Indeed, we show that the kinase activity associated with the p350 component of DNA-PK is potentially functional in *xrs-6* and XR-V15B cells. Our results also demonstrate that a direct consequence of th Ku defect is a concomitant deficiency in the kinase activity of DNA-PK. These data with mutant cells thus suggest strongly that Ku/DNA-PK functions as a complex *in vivo*, as it does *in vitro*, and indicate that kinase activity requires Ku. Furthermore, our results suggest that no other endogenous proteins can target p350 to dsDNA and cooperate with p350 to yield DNA-PK activity.

xrs-6 cells are sensitive specifically to agents that induc DNA double-strand breaks (19, 20). Thus, the DNA damage sensitivity profile of *xrs-6* cells matches the fact that DNA-PK is activated *in vitro* by dsDNA ends (1, 2, 4, 8) and suggests that DNA-PK functions in DNA repair by recognizing such structures. The observation that immunoglobulin V(D)J site-specific recombination is also defective in *xrs-6* cells additionally implicates DNA-PK in this process. Consistent with this, the V(D)J recombination defect of *xrs-6* cells is not in generating site-specific DNA double-strand cuts but in subsequent steps that may overlap with the repair of dsDNA lesions that ar generat d by mutag nic agents (19).

Our results raise the intriguing possibility that the p350 catalytic component of DNA-PK executes important functions

in DNA repair and recombination. One way to verify this will be to determine wheth r mutants defectiv in p350 ar impaired in DNA double-strand break repair. It will clearly be of great interest to determine whether p350 is defective in cell lines such as XR-1 and V3/*scid*, which are impaired in DNA doubl -strand break repair and V(D)J recombination but bel ng t different c mplementation groups from *xrs-6* (refs. 33–35 and references therein). Unfortunately, the gene encoding p350 has not yet been cloned fully, and this is proving t be an arduous task since it is a large gene, which spans more than 100 kb of genomic DNA.

There are several mechanisms (not necessarily mutually exclusive) by which DNA-PK may function in DNA repair and recombination. One is that the binding of Ku/DNA-PK to DNA termini protects them from nuclease digestion or assists in the alignment of two adjacent broken ends. In line with a role in protecting DNA ends from nucleases, the rare V(D)J recombination products that are generated in *xrs-6* cells frequently bear large deletions, presumably as a result of nuclease attack (21). Such DNA end protection could just require Ku but might also require the entire DNA-PK complex containing p350. Another possibility is that, when bound to DNA ends, DNA-PK phosphorylates and activates components of the DNA repair and recombination apparatus and thus restricts their activities to the appropriate cellular location. A third possibility is that DNA-PK enhances repair indirectly by phosphorylating and inactivating transcription factors to allow access by the repair and recombination machinery. Finally, it is possible that DNA-PK functions as a DNA-end sensor and that its activation initiates a signaling pathway to alert the cell to the fact that it has sustained DNA damage (36). The availability of cell lines deficient in DNA-PK should prove extremely useful in examining these exciting possibilities.

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